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(54) Title: IMPROVED PSEUDOTYPED RETROVIRUSES

(57) Abstract: Pseudotyped retroviruses having viral glycoproteins with modified O glycosylation regions are provided. Also provided are methods for making the pseudotyped retroviruses of the present invention and for using the pseudotyped retroviruses for transduction of target cells. Cells for stably producing the pseudotyped retroviruses or the present invention are also provided.

IMPROVED PSEUDOTYPED RETROVIRUSES

This application claims the benefit of U.S. Provisional Application Serial No. 60/386,064, filed 04 June 2002, and of U.S. Provisional Application Serial No. 60/458,070, filed 27 March 2003, each of which is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

This invention relates generally to pseudotyped viruses and methods of use of the viruses. Specifically, the invention relates to retroviruses pseudotyped with glycoproteins in which an *O*-glycosylation region has been modified, and use of these viruses for gene transfer and gene therapy.

BACKGROUND OF THE INVENTION

Gene therapy is one of the fastest growing areas in experimental medicine. However, most studies are only Phase I or Phase II clinical studies designed mainly to evaluate the toxicity of the viral vectors and constructs being used. A major drawback has been the design of vectors that are both safe and efficacious. Recent efforts in the field have been directed toward the use of retroviral vectors and viral vectors pseudotyped with glycoproteins from highly virulent viruses such as filoviruses.

Retroviruses are ribonucleic acid (RNA) viruses that include an RNA genome enclosed within a viral capsid wherein the capsid is surrounded by an envelope, or lipid bilayer. Glycoproteins present in the lipid bilayer interact with receptors on the surface of various host cells and allow the retroviruses to enter the host cell. Once in the cell, the retroviruses reverse transcribe the RNA of the viral genome into a double–stranded DNA and incorporate the DNA into the cellular genome as a provirus. Gene products from the integrated foreign DNA may then be produced so that progeny viral particles may be assembled. As retroviruses can be modified to carry exogenous nucleotide sequences of interest, such recombinant retroviruses have a variety of uses. For example,

such recombinant retroviruses are important in introducing desired exogenous sequences into a cell, so that relatively high levels of the protein encoded by the sequences may be produced. However, use of such recombinant retroviruses has several drawbacks.

One such drawback is that retroviruses do not have a broad host range. Efforts at increasing the host range of retroviruses have included substituting the envelope glycoproteins of the retrovirus with that of a different virus, thus forming a pseudotyped retrovirus. The pseudotyped retrovirus advantageously has the host range of the different virus. However, some retroviruses have been pseudotyped with viral glycoproteins that are toxic to cells, so the cells can only produce the virus for a limited time. Furthermore, in many cases, the pseudotyped retroviruses cannot be stably produced and may not be produced at a high titer. Stable cell lines have been developed to overcome the toxicity problems and to stably produce such pseudotyped retroviruses. However, there still exists a need for pseudotyped retroviruses that will allow for the production of high titers that would be required for routine gene transfer and/or gene therapy.

Thus it would be desirable to have a pseudotyped retrovirus that is not toxic to cells and produces high titers of a competent virus. It would also be desirable to have a cell line to produce such retroviruses. Methods for using such a virus would also be desirable.

SUMMARY OF THE INVENTION

It has been discovered that deleting the *O*-glycosylation region of a viral glycoprotein of a pseudotyped retrovirus allows for stable production of the pseudotyped virus from various cell lines. Pseudotyped retroviruses containing viral glycoproteins with a deleted *O*-glycosylation region were produced in higher titer than those with wild-type viral glycoproteins, and were also more efficient in transducing target cells. Accordingly, one aspect of the invention provides pseudotyped retroviruses that include recombinant RNA surrounded by, enclosed within, or otherwise associated with a retroviral core that includes a viral capsid, matrix and nucleocapsid. Surrounding the retroviral capsid is a lipid bilayer that includes least one viral glycoprotein disposed therein. The *O*-

glycosylation region of the glycoprotein disposed in the lipid bilayer has been modified through deletion, in whole or in part, or mutation. In one embodiment, the viral glycoprotein is a filoviral glycoprotein such as, but not limited to, Ebola virus or Marburg virus glycoprotein. The recombinant RNA preferably contains a nucleotide sequence that defines a selected biomolecule intended for delivery to the target cell. The nucleotide sequence can encode a protein to be expressed in the target cell, or it can function as a bioactive RNA in the target cell.

In another aspect, the invention provides producer cells for producing pseudotyped retroviruses having a viral glycoprotein in which the *O*-glycosylation region is deleted, in whole or part. Accordingly, the present invention provides eukaryotic producer cells that include nucleotide sequences encoding retroviral proteins Gag polypeptide, Pro polypeptide, and Pol polypeptide and a fourth nucleotide sequence encoding at least one viral glycoprotein in which the *O*-glycosylation region of the glycoprotein has been modified. Preferably, the fourth nucleotide sequence encodes a filoviral glycoprotein, such as, for example, a Marburg virus or Ebola virus glycoprotein having a modified *O*-glycosylation region as described herein. In a preferred form of the invention, the cells stably produce inventive pseudotyped retroviruses.

In a further aspect of the invention, methods for introducing nucleotide sequences into a target cell using the pseudotyped viruses of the present invention are provided. The viruses can be used to introduce a nucleotide sequence into a cell in cell culture, *ex vivo* or *in vivo*. The pseudotyped viruses of the invention are useful for introduction of a nucleotide sequence into a wide range of cell types.

In yet another aspect of the invention methods are provided for producing high titers of pseudotyped retroviruses. The methods involve introducing into a recombinant producer cell nucleotide sequences that encode a viral glycoprotein in which the *O*-glycosylation region is modified. Also present in the cells are nucleotide sequences that encode for other proteins necessary to produce a pseudotyped retrovirus in high titers and that is more efficient in transfecting target cells.

Additional objects, advantages, and features of the present invention will become apparent from the following description, taken in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A is schematic representation of the Ebola virus glycoprotein showing the GP_1 and GP_2 subunits of the glycoprotein drawn to scale with residue numbers indicated below the diagram.

Figure 1B shows the nucleotide sequence (SEQ ID NO:2) and restriction map of plasmid pEboGPΔ309-489. The sequence of the modified Ebola glycoprotein begins with the ATG start codon at nucleotides 956-958 and ends with the TAG stop codon at nucleotides 2447-2449. The *O*-glycosylation region represented by wild-type codons 309-489 has been deleted in this construct. Codon 308 begins at nucleotide 1877 and codon 490 begins at nucleotide 1886. The insertion sequence between codons 308 and 490 (TCTAGA at nucleotides 1880-1886) encodes the dipeptide Ser-Arg.

Figure 1C shows a portion of the nucleotide sequence (SEQ ID NO:1) and restriction map of plasmid pEboGP showing the complete coding sequence of Ebola glycoprotein. The coding sequence begins with the ATG start codon at nucleotides 956-958 and ends with the TAG stop codon at nucleotides 2984-2986. The *O*-glycosylation region represented by wild-type codons 309-489, which is deleted in pEboGPΔ309-489, begins at nucleotide 1880 and ends at nucleotide 2422.

Figure 2 is a western blot showing the expression and incorporation of the Δ308-489 Ebola GP into pseudotyped retrovirus.

Figure 3 is a western blot showing the extent of *O*-glycosylation of the Δ308-489 Ebola GP incorporated into pseudotyped retroviruses.

DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

It has been discovered that deleting the *O*-glycosylation region of a viral glycoprotein of a pseudotyped retrovirus allows for stable production of the pseudotyped virus from producer cell lines. Pseudotyped viruses having viral glycoproteins with a deleted *O*-glycosylation region were produced in higher

titer than those with wild-type viral glycoproteins and were also more efficient in transducing target cells.

Accordingly, one aspect of the invention provides a pseudotyped retrovirus. The pseudotyped retrovirus is formed from retroviral components that include a retroviral core surrounded by a lipid bilayer, and a recombinant RNA. Disposed in the lipid bilayer is at least one non-wild-type viral glycoprotein which contains a modified *O*-glycosylation region. The modification can take the form of a deletion of the *O*-glycosylation region from the viral glycoprotein, in whole or in part, or mutation of the *O*-glycosylation region. The resulting pseudotyped retrovirus exhibits increased transduction capacity and expanded tropism, compared to the wild-type retrovirus.

In one embodiment, the viral glycoprotein is a filoviral glycoprotein such as, but not limited to, Ebola virus or Marburg virus glycoprotein.

It has been discovered that eukaryotic producer cells may be constructed that stably produce pseudotyped retroviruses having a viral glycoprotein disposed in their lipid bilayer wherein the *O*-glycosylation region of the viral glycoprotein has been modified. Modification of the *O*-glycosylation region results in enhanced glycoprotein processing and incorporation into retroviral particles, allowing for stable production of the pseudotyped viruses.

It has further been discovered that levels of viral transduction into target cells are significantly higher for the pseudotyped retroviruses having glycoproteins with modified *O*-glycosylation regions as compared to pseudotyped retroviruses bearing wild-type glycoproteins.

Glycoprotein for use in retroviral pseudotyping

The present invention contemplates that the *O*-glycosylation region of the glycoprotein used to pseudotype the retrovirus is modified, either by deletion (in whole or part) or mutation. Deletion of the *O*-glycosylation region allows the pseudotyped retrovirus to be stably produced in the recombinant virus producer cells. By "stable production" or "stably produced", it is meant that the producer cells will produce pseudotyped retroviruses indefinitely (i.e., during the life span of the cell). While not wishing to be bound by theory, it is thought that deletion of the *O*-glycosylation region allows for increased

expression of processed glycoprotein in the recombinant virus producer cell. This glycosylation step may be rate limiting during production of the virus, and glycosylation of high amounts of viral glycoproteins may be toxic to the producer cells. Thus, increased expression may be a result of bypassing the post-translation glycosylation step. Increased transduction is observed and may be due to the greater quantity of virus produced.

The viral glycoprotein used in pseudotyping the retrovirus of the invention may be any viral glycoprotein having an *O*-glycosylation region. An *O*-glycosylation region can be identified as one that is rich in proline, serine and threonine residues. Such regions can be discovered by searching protein databases for potential *O*-glycosylation regions using computer algorithms such as NetOGlyc v. 2.0 (J.E. Hansen et al., Glycogonjugate J., 15:115-130, 1998; J.E. Hansen et al., Nucleic Acids Research, 25, 278-282, 1997; J.E. Hansen et al. Biochemical Journal, 308, 801-813, 1995). Alternatively, *O*-glycosylation regions can be identified biochemically, for example by using antibodies to perform western blots.

Filoviral proteins, such as those of Ebola virus (e.g., Ebola Zaire [complete genome, GenBank Acc. No. NC 002549; glycoprotein, GemBank Acc. No. U23187], Ebola Reston [complete genome, GenBank Acc. No. NC 004161; glycoprotein, GenBank Acc. No. U23152] and Ebola Sudan [glycoprotein, GenBank Acc. No. U23069]; see Fig. 1) and Marburg virus [complete genome, GenBank Acc. No. NC 001608; glycoprotein, GenBank Acc. No. Z12132]), have *O*-glycosylation regions and infect a broad spectrum of mammalian hosts. By way of non-limiting example, the *O*-glycosylation region for the Ebola glycoprotein (Zaire) is from about nucleotide 309 to nucleotide 489 of SEQ ID NO:1. The nucleotide sequences encoding the filoviral glycoproteins may be obtained as described in Sanchez et al. (1993) *Virus Res.* 29 (3):215-240 and Will et al., (1993) *J. Virol.* 67:1203-1210. It is expected that other viruses not specifically mentioned herein and having glycoproteins of similar structure to the filoviral glycoproteins may be advantageously used in the present invention.

Viral glycoproteins from filoviruses facilitate the introduction of genes into many different cell types and are therefore preferred for use in the

invention. As an example, Ebola glycoprotein-pseudotyped lentiviruses have specificity in particular for transducing airway epithelia cells from the apical surface and therefore hold promise for reagents for gene therapy for diseases of the lung such as cystic fibrosis.

It will be appreciated by the skilled artisan that the *O*-glycosylation region can be modified by deleting it either in whole or part from the glycoprotein. Deletion involves removing part or all of the nucleotide sequence encoding the region. In embodiments wherein only part of the *O*-glycosylation region is deleted, a sufficient number of nucleotides is deleted to cause the transduction level in a target cell to increase by at least 2-fold, preferably at least 5-fold, and more preferably at least 10-fold, 20-fold, 50-fold or 100-fold. Transduction levels can be readily determined by using the assay described in Example I.

Alternatively, the nucleotide sequence can mutated, for example by modifying it to replace the codons for serine and threonine, the amino acids that are *O*-glycosylated, with codons for conservative amino acid substitutions such as alanine which will not be *O*-glycosylated. A sufficient number of serine and/or threonine residues are mutated such that transduction level into a target cell is increased as described above.

Retroviral particle

The pseudotyped retrovirus of the invention is a recombinant retroviral particle that includes recombinant RNA surrounded by, enclosed within, or otherwise associated with a retroviral core that includes a viral capsid, matrix and nucleocapsid. A viral glycoprotein, modified to delete or mutate the *O*-glycosylation region, is disposed within a lipid bilayer which surrounds the retroviral core.

The recombinant RNA includes both coding and noncoding retroviral control elements. The term "nucleotide sequence", or "nucleic acid sequence" as used herein is intended to refer to a natural or synthetic linear and sequential array of nucleotides and/or nucleosides, and derivatives thereof. The terms "encoding" and "coding" refer to the process by which a nucleotide sequence, through the mechanisms of transcription and translation, provides the

information to a cell from which a series of amino acids can be assembled into a specific amino acid sequence to produce a polypeptide.

Nucleotide sequences required for retroviral transcription, reverse transcription and integration in the target cell are thus included in the recombinant RNA, as well as elements required for incorporation (packaging) of the retrovirus particle (e.g., psi). Reverse transcriptase and integrase give the retrovirus the ability to incorporate a gene encoding a desired protein into a genome of a target cell after the retrovirus contacts, or is incubated with, the cell. The pseudotyped retrovirus may include other proteins, in addition to integrase, that aid its stable integration into the chromosomes of a target cell. For example, with respect to a lentivirus, the pseudotyped retrovirus may include proteins such as vpr, vif and vpu. Other sequences known to the art that are useful for transducing genes may also be present in the recombinant RNA.

An illustrative, nonlimiting example of the structural organization of a recombinant RNA forming a part of the pseudotyped virus of the invention is a nucleotide sequence that begins with an optional 5' long terminal repeat (LTR) or other transcription and/or integration element(s), followed by a primer binding site (PBS) that initiates reverse transcriptase binding, followed by a viral packaging sequence such as psi, which packaging sequence is optionally flanked by splice acceptor and splice donor sites, followed by a reverse transcriptase initiation site for the second strand, followed by an optional 3' LTR. When the recombinant RNA includes a nucleotide sequence that encodes a desired protein or a bioactive RNA (see below), that sequence is typically located between the splice donor site and the reverse transcriptase initiation site.

The pseudotyped retrovirus of the invention can be replicative or nonreplicative. In most applications, it is desirable that the retrovirus be nonreplicative. Replication incompetent retroviruses typically typically lack one or more of the Gag, Pol, Pro or Env proteins.

The recombinant RNA further optionally includes a nucleotide sequence that defines a selected biomolecule intended for delivery to the target cell. The nucleotide sequence can encode a protein to be expressed in the target cell, or it can function as a bioactive RNA in the target cell. This protein or RNA is produced by a target cell upon introduction of the pseudotyped retrovirus into

the target cell. The protein (or RNA) can serve as a detectable marker, or can provide a beneficial or therapeutic effect if introduced into an animal, as described in more detail below.

Recombinant virus producer cells

The invention provides recombinant virus producer cells, preferably eukaryotic cells forming eukaryotic cell lines, that contain nucleotide sequences encoding retroviral Gag polypeptide, retroviral Pro polypeptide, retroviral Pol polypeptide and at least one viral glycoprotein, such as a filoviral glycoprotein, in which the *O*-glycosylation region of the glycoprotein has been deleted or modified, as described above. (Note that *pro* and *pol* often occur so close together on a viral genome that the term *pol* is often used to refer to both *pro* and *pol*). The eukaryotic cell line is grown *in vitro* to produce the pseudotyped retroviruses.

Any of a wide variety of cells can be selected for *in vitro* production of a pseudotyped retrovirus according to the invention. Eukaryotic cells are preferred, particularly mammalian cells including human, simian, canine, feline, equine and rodent cells. Human cells are most preferred. It is further preferred that the cell be able to reproduce indefinitely, and is therefore immortal. Examples of cells that may be advantageously used in the present invention include NIH 3T3 cells, COS cells, Madin-Darby canine kidney cells, human embryonic 293T cells and any cells derived from such cells, such as gpnlslacZ ϕNX cells, which are derived from 293T cells and described in the Examples. Highly transfectable cells, such as human embryonic kidney 293T cells, are preferred. By "highly transfectable" it is meant that at least about 50%, more preferably at least about 70% and most preferably at least about 80% of the cells can express the genes of the introduced DNA.

The retroviral *gag*, *pro* and *pol* nucleotide sequences and other retroviral nucleotide sequences needed for forming the specified pseudotyped retroviruses may be obtained from a wide variety of genera in the family Retroviridae, including, for example, Alpharetrovirus (e.g., avian leukosis virus), Betaretrovirus (e.g., mouse mammary tumor virus), Gammaretrovirus (e.g., murine leukemia virus and Moloney monkey leukemia virus [Mo-MuLV]),

Deltaretrovirus (e.g., bovine leukemia virus), Epsilonretrovirus (walleye dermal sarcoma virus), Lentivirus (e.g., humanimmunodeficiency virus I, feline immunodeficiency virus and Chimpanzee foamy virus) and spumavirus. Retroviral sequences are preferably obtained from the Moloney murine leukemia virus (Mo-MuLV). Such sequences are well known in the art. For example, nucleotide sequences encoding MMLV Gag, Pro and Pol polypeptides may be found in Shinnick et al., *Nature*, 293:543-548 (1981).

Depending on the application, the retroviral components of the pseudotyped retrovirus of the invention are preferably derived from a simple retrovirus such as MoMuLV, or a more complicated retrovirus such as a lentivirus. Examples of lentiviruses include feline immunodeficiency virus (FIV), human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV) and equine infectious anemia virus (EIAV). Unlike most other retroviruses, lentiviruses have the capacity to integrate the genetic material they carry into the chromosomes of non-dividing cells as well as dividing cells. Therefore, lentiviral nucleotide sequences encoding proteins that allow for chromosomal integration of virally transported nucleic acid in non-dividing cells are advantageously employed, as the host range of the pseudotyped retroviruses will be broadened.

Suitable retroviruses, including those described above, are readily publicly available from the American Type Culture Collection (ATCC) and the desired nucleotide sequences may be obtained from these retroviruses by methods known to the skilled artisan. For example, the nucleotide sequences may be obtained by recombinant DNA technology. Briefly, viral DNA libraries may be constructed and the nucleotide sequences may be obtained by standard nucleic acid hybridization or polymerase chain reaction (PCR) procedures, using appropriate probes or primers. Alternatively, supernatant medium from cells infected with the respective virus can be isolated and the desired retroviral nucleotide sequences may be amplified by PCR. Such vectors may also be constructed by other methods known to the art.

It is preferred that the *gag, pro* and *pol* nucleotide sequences are contiguous to each other as found in native retroviral genomes, such as in the order 5'-gag-pro-pol-3'. It is further preferred that these retroviral nucleotide

sequences are chromosomally-integrated into the cellular genome of the producer cell. Furthermore, the gag-pro-pol nucleotide sequences are preferably operably linked at the 5' end of the *gag* nucleotide sequence to a promoter sequence, so that transcription of the sequences may be achieved.

A nucleic acid sequence is "operably linked" to another nucleic acid sequence, such as a promoter sequence, when it is placed in a specific functional relationship with the other nucleic acid sequence. The functional relationship between a promoter and a desired nucleic acid typically involves the nucleic acid and the promoter sequences being contiguous such that transcription of the nucleic acid sequence will be facilitated. Two nucleic acid sequences are further said to be operably linked if the nature of the linkage between the two sequences does not interfere with the ability of the promoter region sequence to direct the transcription of the desired nucleotide sequence, or interfere with the ability of the desired nucleotide sequence to be transcribed by the promoter sequence region. Typically, the promoter element is generally upstream (i.e., at the 5' end) of the nucleic acid coding sequence.

A wide variety of promoters are known in the art, including cell-specific promoters, inducible promoters, and constitutive promoters. The promoters may further be selected such that they require activation by activating elements known in the art, so that production of the protein encoded by the specified nucleic acid sequence may be regulated as desired. It is well within the purview of a person skilled in the art to select and use an appropriate promoter in accordance with the present invention. For example, the promoters that may be advantageously present in the cell, 5' to the gag-pro-pol sequences, include the rat actin promoter and the MMLV promoter. Furthermore, the cytomegalovirus (CMV) promoter has been found to be an excellent promoter in the inventive system.

Other regulatory elements, such as enhancer sequences, cooperate with the promoter and transcriptional start site to achieve transcription of the nucleic acid insert coding sequence, may also be present in the cell 5' to the nucleotide sequences that encode retroviral proteins. By "enhancer" is meant nucleotide sequence elements which can stimulate promoter activity in a cell, such as a bacterial or eukaryotic host cell.

Another aspect of the invention provides methods of making eukaryotic cells for producing pseudotyped retroviruses. The method includes introducing into the cells described above the nucleotide sequences described above, i.e., those encoding the retroviral Gag, Pro and Pol polypeptides, and those encoding a filoviral glycoprotein in which the *O*-glycosylation region is deleted or mutated, in part or in whole, into the cell.

The nucleotide sequences may be introduced into the desired cell utilizing any of variety of vectors known to the skilled artisan. For example, plasmid vectors, cosmid vectors, and other viral vectors, such as retroviral vectors, may be used. It is preferred that the nucleotide sequences encoding the Gag, Pro and Pol polypeptides are on a vector separate from the nucleotide sequences encoding the viral glycoproteins.

In one mode of practicing the invention, plasmid vectors are advantageously used to introduce, or transfect, the nucleotide sequences into the selected cell. A wide variety of plasmid vectors may be used, including pTRE, pCMV-Script and pcDNA3, although pcDNA3 is a preferred vector. The *gag, pro* and *pol* nucleotide sequences are preferably on the same plasmid, and, as discussed above, are preferably contiguous to each other. However, the skilled artisan is aware that other spatial configurations of the nucleotide sequences may be utilized when constructing the plasmids. The vector also preferably includes a promoter 5' to, or upstream from, the *gag* nucleotide sequence. The vectors may further include other regulatory elements, such as enhancer sequences, as discussed above.

The nucleotide sequences encoding the viral glycoproteins are preferably, but need not be, on a plasmid, or other vector, separate from the *gag, pro* and *pol* nucleotide sequences. The viral glycoprotein encoding sequences, such as the sequences encoding the filoviral glycoproteins are also preferably operably linked to a promoter sequence described above. In the case of viral glycoproteins that are formed from separate polypeptides, the two polypeptides may, if desired, be supplied on different plasmids. It is preferred, however, that such sequences are on the same vector, and preferably contiguous to each other so they will be transcribed utilizing the same promoter. In one preferred form of the invention, the promoter sequence is a cytomegalovirus promoter sequence.

Plasmids, or other vectors carrying the nucleotide sequences encoding the viral glycoproteins, may also include other regulatory elements, such as enhancers, as described above.

The vectors may be introduced into the cells in a variety of ways known to the skilled artisan, for example, discussed in *Current Protocols in Molecular Biology,* John Wiley and Sons, edited by Ausubel et al. (1988) and Sambrook et al., *Molecular Cloning, A Laboratory Manual,* Cold Spring Harbor Laboratory Press (2000). For example, vectors may be transfected into a cell by a calcium phosphate precipitation method. Other methods for introduction of the vectors include, for example, electroporation and lipofection.

In order for the producer cells to produce the pseudotyped retroviruses of the invention, they must express the recombinant RNA that is ultimately packaged into the retroviral particles. The producer cells must therefore be supplied with the recombinant RNA. The recombinant RNA may be introduced into the producer cell as DNA or as RNA, in any of a variety of ways known to the skilled artisan. Plasmids are typically used to introduce DNA encoding the recombinant RNA into the producer cells. Alternatively, defective retroviruses (i.e., those which do not have the capability to produce all of the viral proteins necessary for production of a retrovirus having the ability to infect a cell and produce progeny viruses) can be utilized to cause genomic integration of the DNA encoding the recombinant RNA into the producer cell's genome.

Pseudotyped retrovirus as a vehicle for delivery of genetic material

In many cases, one may wish to quickly visually detect those cells which have taken up a vector and that produce a specified protein from the vector. The desired protein encoded by the retroviral recombinant RNA may accordingly be one that facilitates detection of entry of the pseudotyped retrovirus into a target cell. The desired protein may be, for example, a visually detectable component, or marker, such as one that emits visible wavelengths of light, or one that can be reacted with a substrate to produce color of specified wavelengths or other detectable reaction. For example, such nucleotide sequences include the nucleotide sequence encoding the fluorescent proteins such as green, blue and red fluorescent proteins (e.g., *A. victoria* green fluorescent protein (GFP);

nucleotide sequences listed in Prasher et al., *Gene* 111:229 (1992)) and the LacZ gene (produces β-galactosidase, which reacts with a well-known substrate, X-gal), both of which are well known in the art and may be obtained commercially. Cells that have taken up the vector and express the nucleotide sequences encoding a protein may be identified and separated from cells that do not express the sequences by a fluorescence-activated cell sorting procedure as known in the art.

The invention further provides a screening kit for use in evaluating transduction efficiency of a retrovirus in selected target cells. The screening kit includes a pseudotyped retrovirus of the invention, appropriate buffers, and instructions for use.

Advantageously, expression of a detectable marker allows viral entry into a target cell to be confirmed. Expression of the detectable marker can also be used to ensure that the pseudotyped retroviruses that are formed are replication incompetent (i.e., do not have all the sequences necessary in their viral genome to produce progeny retroviruses). Supernatant isolated from target cells transduced by the pseudotyped retroviruses and contacted with a second target test cell should not result in localization of the fluorescent protein in the second target test cell because there should be no retroviruses in the supernatant. A lack of retroviruses in the supernatant would indicate that no replication competent viruses are produced inside the initial target cell, so none of those initial target cells serves as a source of competent retroviruses. Even though it does not make new virus particles, the initial target cell nonetheless expresses the marker because the marker is introduced into its genome via the pseudotyped retrovirus with which it was transduced.

In addition or alternatively to the detectable marker, the pseudotyped retroviruses of the invention may include recombinant RNA that operably encodes a protein that is needed by an animal, either because the protein is no longer produced, is produced in insufficient quantities to be effective in performing its function, or is mutated such that it either no longer functions or is only partially active for its intended function. Other examples of nucleotide sequences that can be included in the retroviral recombinant RNA include those that encode a protein or bioactive RNA (typically a ribozyme) that is effective

to treat a viral infection, stimulate the hosts immune system, or preferentially infect and kill cancer cells in the host.

The present invention further includes methods of introducing desired nucleotide sequences into a target cell using a pseudotyped retrovirus. A target cell is eukaryotic and can be a vertebrate or invertebrate cell. Examples of eukaryotic cells include cells from insects, birds, fish and mammals. Preferably the target cell is a mammalian cell, more preferably a feline, canine, bovine, or human cell. The mammalian cell can be a normal cell or a transformed, cancerous or precancerous cell. The target cell may be a cell already infected by a virus.

In one embodiment, the method of delivering the nucleotide sequences to the target cell includes contacting, or transducing, a target cell permissive for filoviral entry, with a retrovirus that has been pseudotyped with a filoviral glycoprotein as described above that includes the desired nucleotide sequence in its genome. The cells can be contacted while present in the organism (*in vivo*) (Sharkey et al., *J Virol*, 75:2653-2659 (2001)), while explanted and outside the organism (*ex vivo*), or in cell culture (Sinn et al., *J Virol* 2003 May 15;77(10):5902-10 and Kang et al., *J. Virol.*, 76:9378-9388 (2002)). Cells contacted *ex vivo* can, if desired, be reimplanted into the organism. *In vivo* delivery of the pseudotyped retrovirus can be accomplished using methods well known to the art of therapeutic delivery of genetic material, such as injection, perfusion, aerosolization followed by inhalation, and the like. Dosages can be readily determined by reference to existing retroviral gene therapies.

When the nucleotide sequence encodes a desired protein, the target cell is selected so that it allows expression of the selected nucleotide sequence. The level of transduction may be determined by assay methods known to the skilled artisan, and include assaying for the protein of interest encoded by the introduced nucleotide sequences or assaying for the presence of the nucleotide sequences.

Any existing retroviral therapy can be modified according to the invention by including in the lipid bilayer of the retrovirus the modified glycoproteins described herein. Pseudotyping with the modified glycoproteins

increases the efficiency of gene therapy because the transduction levels are significantly increased, making the procedure more economical as well.

Reference will now be made to specific examples illustrating the compositions and methods above. It is to be understood that the examples are provided to illustrate preferred embodiments and that no limitation to the scope of the invention in intended thereby.

Example I. Murine Leukemia Virus Pseudotyped with Ebola Glycoprotein Lacking *O*-Glycosylation Region

MATERIALS AND METHODS

Cell lines and culture conditions. The human kidney cell line 293 (ATCC Number CRL-1573), the mouse embryo cell line NIH 3T3 (CRL-1658), and the 293T-derived ϕNX (second generation retroviral packaging cells) (Grignani et al., *Cancer Res*, 58:14-19 (1998); Pear et al., *Proc. Natl. Acad. Sci. USA*, 90:8392-8396 (1993); Swift et al., p. 10.17.14-10.17.29. In R. Coico (ed.), Current Protocols in Immunology supp. 31. J. Wiley & Sons (1999)) and gpnlslacZ cell lines were cultured in Dulbecco's minimal essential medium (DMEM) containing 10% heat inactivated fetal bovine serum, 2 mM glutamine, 100 units Penicillin G, and 100 μg/ml streptomycin sulfate, with or without 0.25 μg/ml amphotericin B (growth medium). The gpnlslacZ cells produce envelope protein-deficient replication-incompetent Mo-MuLV particles carrying MFG.S-nlslacZ, a retroviral vector encoding a nuclear localizing β-galactosidase (Sharkey et al., *J Virol*, 75:2653-2659 (2001)).

Plasmids and site-directed mutagenesis. A modified version of the plasmid pTM1 was used in transient expression studies of GP sequences using a vaccinia virus-T7 RNA polymerase (VV-T7) system (Elroy-Stein et al., *Proc Natl Acad Sci U S A*, 86:6126-6130 (1989)). The pTM1 vector was modified to remove an ATG codon (within an *Nco*I site) at the beginning of the multiple cloning site by *Nco*I digestion, mungbean nuclease treatment, and ligation of the blunt-ended DNA. This vector, pTM1(Δ*Nco*I), was used to subclone the entire Ebola virus GP open reading frame (ORF). The GP ORF was cleaved from the

buffer) for 30 minutes at room temperature. Membranes were washed as before, then treated with a commercial chemiluminescent substrate solution (Amersham Pharmacia Biotech), according to the protocols provided by the manufacturer. Specific reactivity to GP was visualized by exposing treated blots to X-ray film.

RESULTS

Pseudotyped retroviruses bearing GPs with altered glycosylation. The role of *O*-glycosylation of the Ebola virus GP was examined through analysis of the effects of deleting the region of the protein that is *O*-glycosylated (Jeffers et al., *J Virol* 2002 Dec;76(24):12463-72). Remarkably, processing and viral incorporation of the Δ309-489 GP was greatly enhanced as shown in Figure 2. The migration of the mature GP_1, GP_0 (the glycosylated but uncleaved glycoform), GP_{pre} (the *N*-glycosylated but not *O*-glycosylated uncleaved glycoform) and deglycosylated GP_0 and GP_{pre} forms of the wild-type GP and of the GP_1, GP_0 and deglycosylated GP_1 and GP_0 forms of the Δ309-489 GP is indicated. There was also a corresponding increase of 696 ± 142% in transduction by the Δ309-489 GP pseudotyped viruses as compared to wild type. The absence of an increase in the mobility of the Δ309-489 GP upon sialadase A and endo-*O*-glycosidase treatment provides confirmation that the region of *O*-glycosylation of the glycoprotein has been removed (Figure 3). The migration of the mature GP_1 of the wild-type and Δ309-489 GPs is indicated. In this experiment a glycosylated serum protein possessing a mobility intermediate between those of the wild-type and Δ309-489 GP_1s was detected. The heterogeneous mobility of the PNGaseF-treated proteins is indicative of incomplete removal of *N*-glycosylation.

The effect of deleting the *O*-glycosylation region of GP_1 (Δ309-489) on expression and transduction were striking. This segment, which is rich in proline, serine, and threonine residues is the most variable among the Ebola GPs. Elimination of this mucin-like region results in enhanced GP processing and incorporation into retroviral particles (Figure 2) and consequently higher levels of transduction by the pseudotyped retroviruses. It is possible that the wild-type GP is retained in the Golgi apparatus until all of the serines and

plasmid pGEM-EMGP1 (Sanchez et al., *Virus Res*, 29:215-240 (1993)) by digestion with *Bam*HI and *Dra*I, and the fragment isolated and directionally ligated into the pTM1(Δ*Nco*I) vector cleaved with *Bam*HI and *Stu*I. The resulting clone, pTM1(Δ*Nco*I)-GP, was used as the target DNA for all site-directed mutagenesis reactions. This clone encodes a GP sequence that differs from the wild-type amino acid sequence in a single residue within the membrane spanning sequence (I662V), and for comparative purposes will be referred to as "wild-type sequence". This mutation is present in the original pGEM3Zf(-)-GP clone, but does not appear to affect the processing or function of the GP. GP residue numbering commences with the methionine of the signal sequence and is continuous through the GP_1 and GP_2 sequences.

The GP clone in which the mucin region was deleted (Δ309-489) was generated from two PCR clones linked by an *Xba*I restriction site, which resulted in the replacement of the mucin sequence with two residues (serine-arginine). Mutations in isolated plasmid clones were identified by direct sequencing of mini-prep DNA using dye-terminator cycle sequencing reactions (ABI) analyzed on either an ABI 373 or 377 sequencer. Large-scale preparations for each type of mutated plasmid DNA were made using commercial kits (Promega Corp. or 5 Prime → 3 Prime, Inc.). The DNA was quantified by UV_{260} absorbance readings, and then stored at -70°C until needed. The coding region (*Bam*HI/*Sal*I fragments) from the plasmid pTM1(ΔNcoI)-GP and mutated versions of this DNA were separately ligated into the *Bam*HI/*Xho*I polylinker sites of the vector pcDNA3 (Invitrogen) to yield pEboGP (Fig. 1C) and pEboGPΔ309-489 (Fig. 1B). These plasmids were then cloned in *E. coli*, and plasmid DNA was isolated for use in pseudotyping studies.

Retrovirus pseudotyping and virus transduction assays. Pseudotyped retrovirus particles consisting of the MuLV cores and the Ebola GP in their envelopes were produced by transfecting wild-type or mutated plasmid DNA into gpnlslacZ cells as previously described by Sharkey et al. (*J. Virol.* 75:2653-2659 (2001)). Virus transduction of β-galactosidase activity into NIH 3T3 cells was determined as described by Sharkey et al. (*J. Virol.* 75:2653-2659 (2001)). All data presented are the average of the results of at least three experiments.

Immunoblot analysis of Ebola virus glycoprotein expression, processing, and incorporation into pseudotyped retroviruses. Medium from transfected ϕNX cells (Grignani et al., *Cancer Res* 58:14-19 (1998); Sanchez et al., *J Virol* 72:6442-6447 (1998); Swift et al., p. 10.17.14-10.17.29. In R. Coico (ed.), Current Protocols in Immunology supp. 31. J. Wiley & Sons (1999)) containing recombinant retroviruses were passed through a 0.45 μm filter and centrifuged through a 30% sucrose cushion at 25,000 RPM in a Beckman 50.2-Ti rotor in a Beckman SS-71 centrifuge. The fluid was aspirated from centrifuge tubes and discarded, and the virus pellet was suspended in 100μl of RIPA buffer (140 mM NaCl, 10 mM Tris HCl pH 8.0, 5 mM EDTA, 1% Na deoxycholate, 1% Triton X-100, 0.1% SDS). Cells were treated with lysis buffer (50 mM Tris HCl pH 8.0, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100), and the cell lysates were then centrifuged in a microcentrifuge at 16,100 x *g* for ten minutes. The proteins in the cell lysate and the suspended viral pellet were each precipitated with a final concentration of 4% TCA for two minutes. The precipitated proteins were then centrifuged at 16,100 x *g* in a microcentrifuge for ten minutes. The supernatant fluid was aspirated and discarded. The pellet was suspended in an equal volume of 1 M Tris[hydroxymethyl]-aminomethane and vortexed vigorously. Proteins whose glycosylation was analyzed were treated sequentially with PNGase F, which removes *N*-linked glycosylation, and with both Sialidase A and Endo-*O*-glycosidase (ProZyme, Inc.), which together remove *O*-linked glycosylation, following protocols provided by the supplier. The pellet suspension was then mixed with 1/6 the volume of 300 mM Tris pH 6.8, 60% glycerol (w/v), 4% SDS (w/v), 0.0012% bromophenol blue (w/v), 6% 2-mercaptoethanol (v/v) and boiled for 5 minutes.

Equal amounts of proteins as determined by the Bradford assay were separated by SDS-PAGE (8.5% acrylamide), and electrophoretically blotted onto nitrocellulose membranes. Membrane blots were immersed in reaction buffer (20 mM Tris, 137 mM NaCl, 0.1% Tween 20, pH 7.6) containing 1% bovine serum albumin and incubated overnight at 4°C. Blots were incubated in reaction buffer containing a rabbit-anti-Ebola SGP/GP diluted 1:1000 for 1 hour at room temperature, washed 3 times in reaction buffer, and reacted with a Goat anti-rabbit-horseradish peroxidase conjugate (diluted 1:20,000 in reaction

threonine residues in the mucin region are modified. While not wishing to be bound by theory, it is thought that elimination of this segment may permit more rapid transit through the Golgi apparatus and higher levels of processing to $GP_1$and GP_2 and of cell-surface expression. Increased viral incorporation may also result from a diminution of GP toxicity. It has been reported that the deletion of the *O*-glycosylation region reduces the cytopathic effects of Ebola virus GP expression (Yang et al., *Science*, 279:1034-1037 (1998)). It has also been suggested that the expression of high levels of the wild-type Ebola GP might lead to exhaustion of the cellular glycosylation machinery (Volchkov et al., *Science*, 291:1965-9 (2001)), which is consistent with the present results and present interpretation.

Example II. Expression of Green Fluorescent Protein in Target Cells Transduced with Pseudotyped Retrovirus

A plasmid was constructed based on pcDNA3.1 (Invitrogen) expresses the Ebola virus (Zaire strain) glycoprotein with its *O*-glycosylation region deleted (amino acids 309-489), under control of the cytomegalovirus (CMV) promoter. pcDNA3.1 is similar to pcDNA1 but it also contains zeocin resistance. This plasmid was transfected into human cell lines (gpGFP, Taylor et al., *Mol. Biol. Cell*, 10:2803-2815 (1999)) that have also been transfected with genes encoding the Moloney murine leukemia virus (Mo-MuLV) *gag* and *pol* (including *pro*) genes, the plasmid MFG.S-GFP, and a gene encoding the *A. victoria* green fluorescent protein (GFP).

Recombinant pseudotyped retrovirus recovered from the supernatant medium of such cells was incubated with several different cell lines (a murine cell line, NIH3T3; a human cell line, HeLa; and a hamster cell line, BHK) and was shown to be capable of introducing the gene encoding the green fluorescent protein into the target cells. Transduction levels associated with the modified pseudovirus were 4.7 fold higher than with the retroviruses pseudotyped with unmodified (native) Ebola virus glycoprotein. These recombinant modified Ebola glycoprotein-pseudotyped retroviruses have substantially improved titers

that make *in vivo* gene transfer and gene therapy experiments with such viruses feasible for the first time.

Example III. Feline Immunodeficiency Virus Pseudotyped with Ebola Glycoprotein Lacking *O*-Glycosylation Region

Feline immunodeficiency virus (FIV)-based vectors, which are a non-primate lentivirus vectors, were pseudotyped using envelope glycoproteins (GPs) from the filoviruses Marburg and Ebola virus (Sinn et al., J Virol 2003 May 15;77(10):5902-10). We observed that primary cultures of well-differentiated human airway epithelia were transduced when filovirus GP pseudotyped FIV was applied to the apical surface. Furthermore, by deleting a heavily *O*-glycosylated extracellular region of the Ebola GP, we improved the titer of concentrated vector several fold.

Vector production. The second-generation FIV vector system utilized in this study was reported previously (Johnston et al., *J. Virol.* 73:4991-5000 (1999) and Wang et al., *J. Clin. Inv.* 104:R55-62 (1999)). The FIV vector construct expressed the β-galactosidase cDNA directed by the CMV promoter. EBOΔO (pEZGP 309-489) has been previously described (Jeffers et al., *J. Virol.*, 76:12463-12472 (2002); Example I). Pseudotyped FIV vector particles were generated by transient transfection of plasmid DNA into 293T cells as described previously (Johnston et al., *J. Virol.* 73:4991-5000 (1999)). FIV vector preparations were titered on HT1080 cells at limiting dilutions and these titers were used to calculate the multiplicities of infection (MOIs). In addition, we found that the filoviral glycoprotein conferred enough stability to the lentiviral vector to withstand centrifuge concentration of greater 1000-fold (data not shown); however, we typically concentrated vector 250-fold by centrifugation for *in vitro* experiments.

Deletion of the O-glycosylated region from the extracellular domain of filoviral glycoproteins. An initial strategy for enhancing filoviral pseudotyped FIV-vector titer was to delete an expansive region from the extracellular domain thought to be heavily *O*-glycosylated. By deleting this region, the efficiency of envelope protein synthesis and transport to the cell surface is enhanced

(Example I). This region may be functionally less important than the flanking regions of the protein simply because of little sequence conservation in this region among all filoviral isolates. The deletion of amino acids 309-489 from the Ebola glycoprotein (EBOΔO) resulted in a marked 74-fold increase in titer over the average titer obtained with the wild-type Ebola glycoprotein.

The complete disclosure of all patents, patent applications, and publications, and electronically available material (including, for example, nucleotide sequence submissions in, e.g., GenBank and RefSeq, and amino acid sequence submissions in, e.g., SwissProt, PIR, PRF, PDB, and translations from annotated coding regions in GenBank and RefSeq) cited herein are incorporated by reference. The foregoing detailed description and examples have been given for clarity of understanding only. No unnecessary limitations are to be understood therefrom. The invention is not limited to the exact details shown and described, for variations obvious to one skilled in the art will be included within the invention defined by the claims.

What is claimed is:

1. A pseudotyped retrovirus comprising recombinant RNA associated with a retroviral core surrounded by a lipid bilayer having disposed therein a glycoprotein comprising a modified *O*-glycosylation region, the recombinant RNA comprising (i) a nucleotide sequence defining a selected biomolecule intended for delivery to a target cell, and (ii) retroviral control elements for packaging, reverse transcription and integration of the retrovirus into a target cell.

2. The pseudotyped retrovirus of claim 1 wherein the retroviral core and control elements are from Moloney murine leukemia virus (Mo-MuLV).

3. The pseudotyped retrovirus of claim 3 wherein the retroviral core and control elements are from a lentivirus.

4. The pseudotyped retrovirus of claim 1 wherein the lentivirus is feline immunodeficiency virus (FIV), human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV) or equine infectious anemia virus (EIAV).

5. The pseudotyped retrovirus of claim 1 wherein the glycoprotein is a filovirus glycoprotein.

6. The pseudotyped retrovirus of claim 1 wherein the selected biomolecule is a protein.

7. The pseudotyped retrovirus of claim 1 wherein the selected biomolecule is a bioactive RNA.

8. The pseudotyped retrovirus of claim 1 having a transduction efficiency into target cells of at least 2-fold higher than a retrovirus pseudotyped with the wild-type glycoprotein.

9. A pseudotyped retrovirus comprising recombinant RNA associated with a retroviral core surrounded by a lipid bilayer having disposed therein an Ebola glycoprotein comprising a modified *O*-glycosylation region, the recombinant RNA comprising (i) a nucleotide sequence defining a selected biomolecule intended for delivery to a target cell, and (ii) retroviral control elements for packaging, reverse transcription and integration of the retrovirus into a target cell.

10. The pseudotyped retrovirus of claim 9 wherein the Ebola glycoprotein contains a deletion of nucleotides 309 to 489 in SEQ ID NO:1.

11. The pseudotyped retrovirus of claim 10 wherein the retroviral core and control elements are from Mo-MuLV retrovirus.

12. The pseudotyped retrovirus of claim 10 wherein the retroviral core and control elements are from a lentivirus.

13. A pseudotyped retrovirus pseudotyped with a glycoprotein comprising a modified *O*-glycosylation region, the pseudotyped retrovirus having a transduction efficiency into a target cell of at least 2-fold higher than a retrovirus pseudotyped with the wild-type glycoprotein.

14. A recombinant virus producer cell comprising *gag*, *pro* and *pol* nucleotide sequences and a nucleotide sequence encoding a glycoprotein comprising a modified *O*-glycosylation region.

15. The recombinant virus producer cell of claim 14 wherein the glycoprotein is an Ebola glycoprotein containing a deletion of nucleotides 309 to 489 in SEQ ID NO:1.

16. The recombinant virus producer cell of claim 15 which is a NIH 3T3 cell, COS cell, Madin-Darby canine kidney cell, human embryonic 293T cell or any cell derived therefrom.

17. A method for making a pseudotyped retrovirus comprising supplying a recombinant RNA to the recombinant virus producer cell of claim 12, wherein recombinant RNA comprises (i) a nucleotide sequence defining a selected biomolecule intended for delivery to a target cell, and (ii) retroviral control elements for packaging, reverse transcription and integration of the retrovirus into a target cell, under conditions such that pseudotyped retrovirus is produced.

18. The method of claim 17 wherein supplying the recombinant RNA to the producer cell comprises introducing a DNA encoding the recombinant RNA into the producer cell.

19. The method of claim 17 supplying the recombinant RNA to the producer cell comprises introducing the recombinant RNA into the cell.

20. A method for transducing a target cell comprising contacting a target cell with the pseudotyped retrovirus of claim 1.

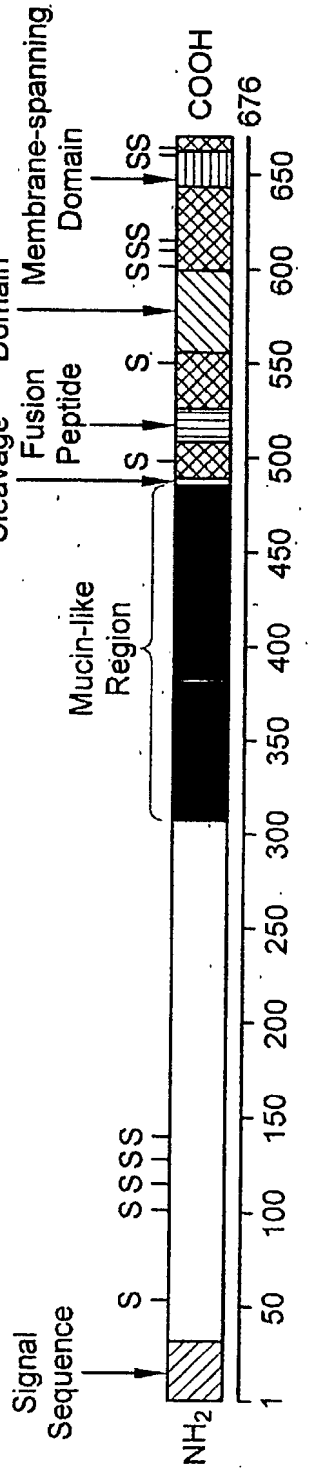
21. The method of claim 18 wherein the target cell is an insect cell, a bird cell, a fish cell or a mammalian cell.

22. The method of claim 19 wherein the target cell is a human cell.

23. The method of claim 18 wherein the cell is *in vivo*, *ex vivo*, or in cell culture.

24. The method of claim 18 wherein the selected biomolecule is a protein, and wherein the transduced target cell expresses the protein.

25. The method of claim 18 wherein the selected biomolecule is a bioactive RNA, and wherein the transduced target cell produces the bioactive RNA.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SS
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NH2
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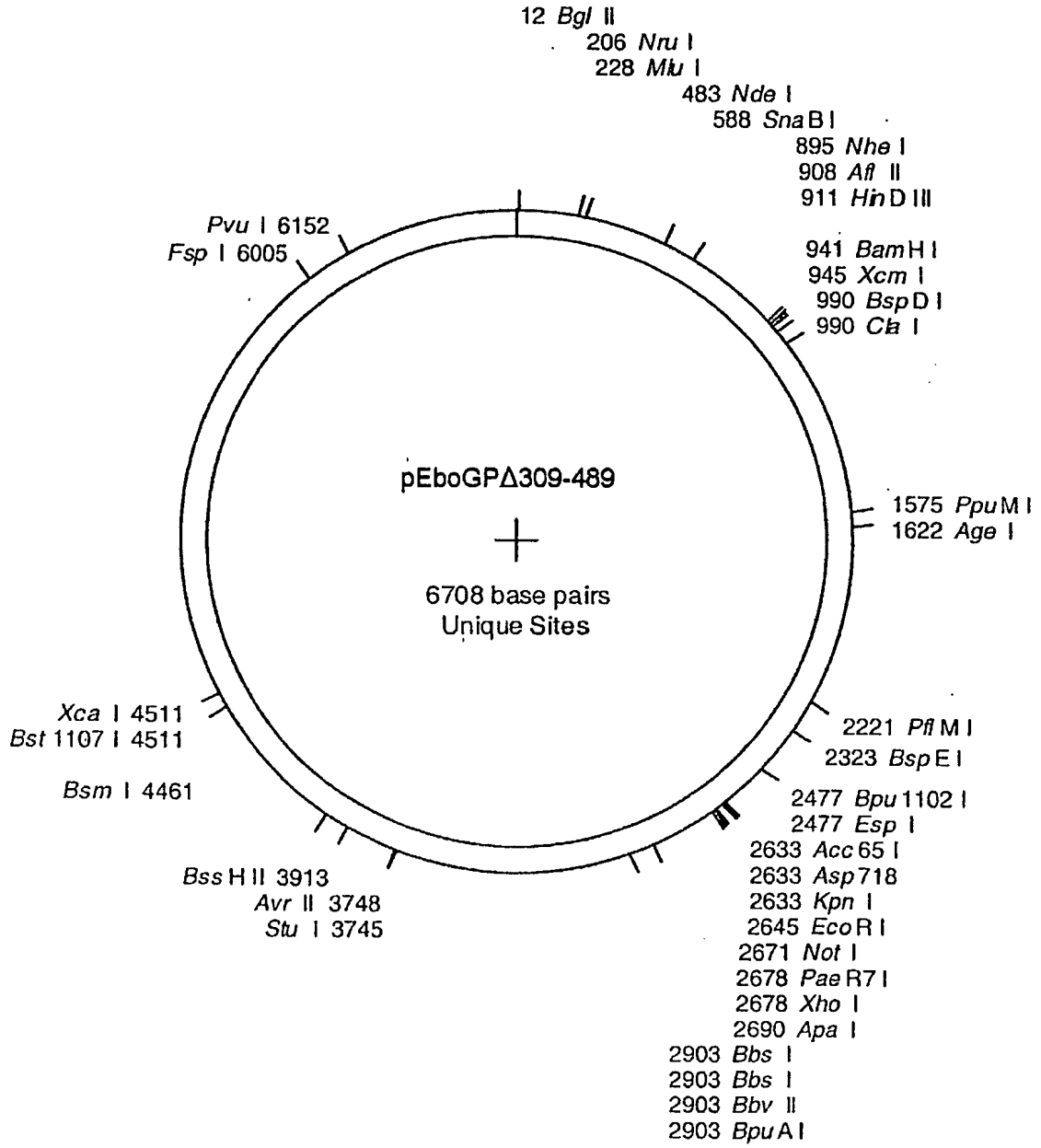
Fig. 1A

Fig. 1B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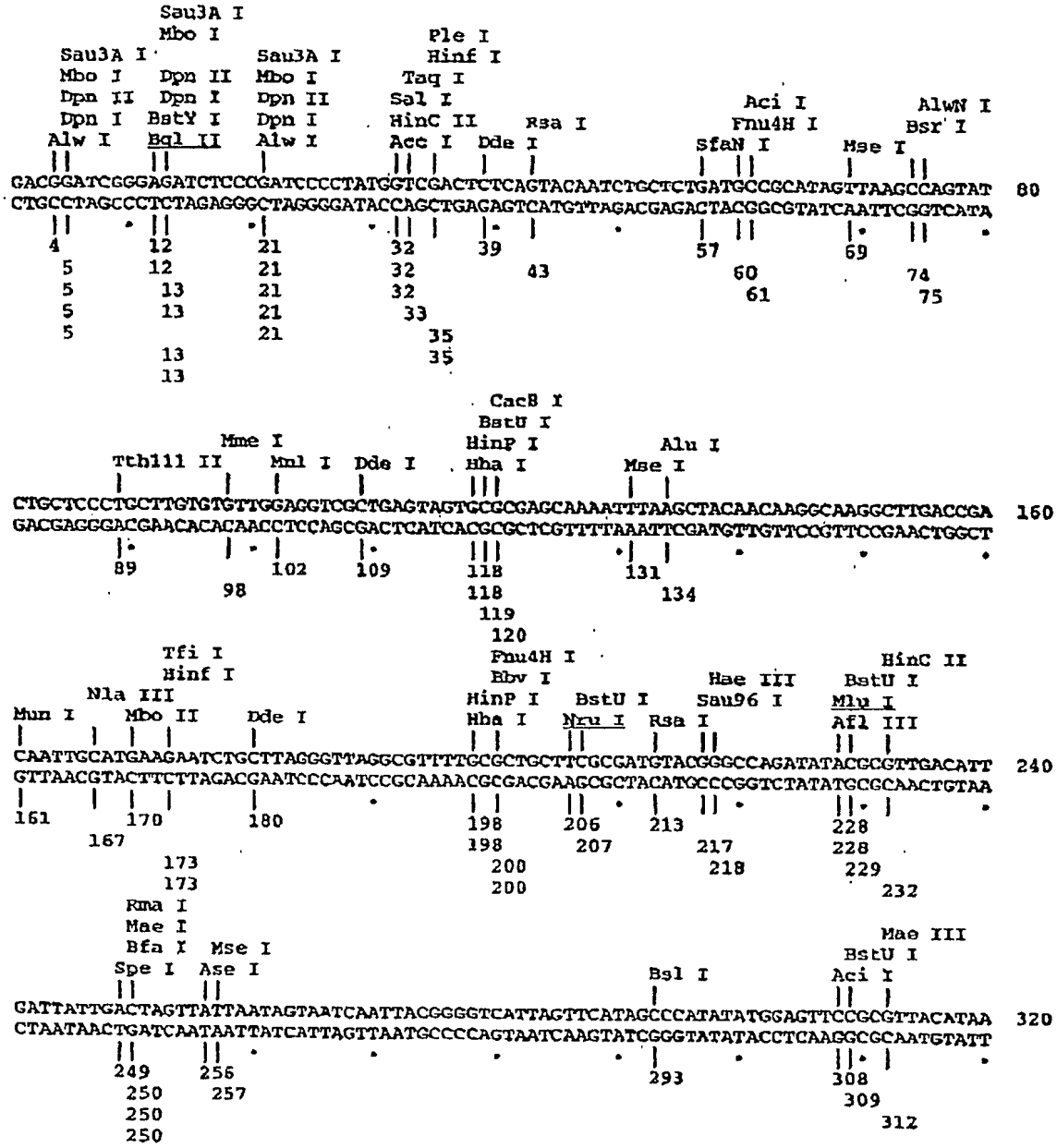
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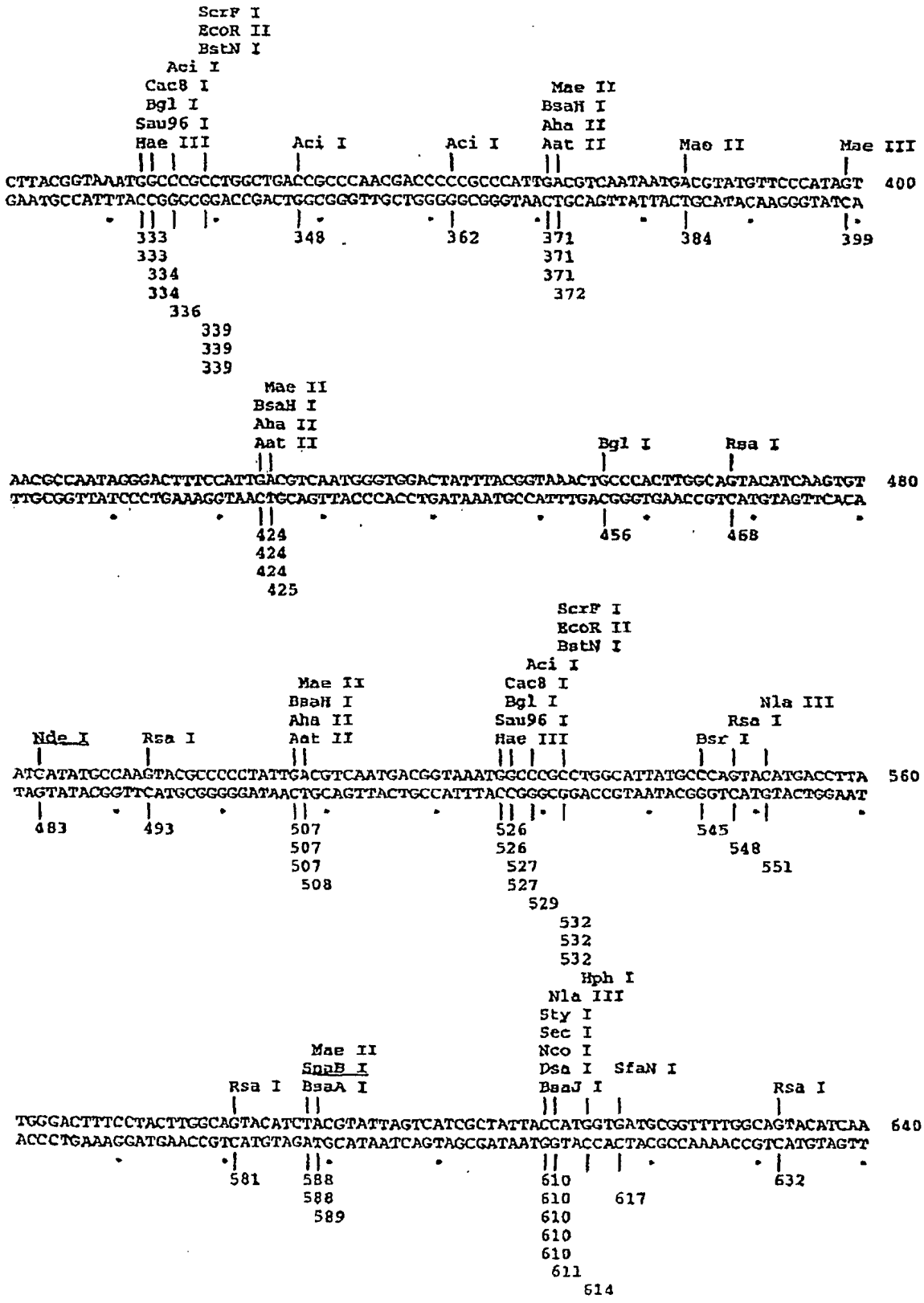
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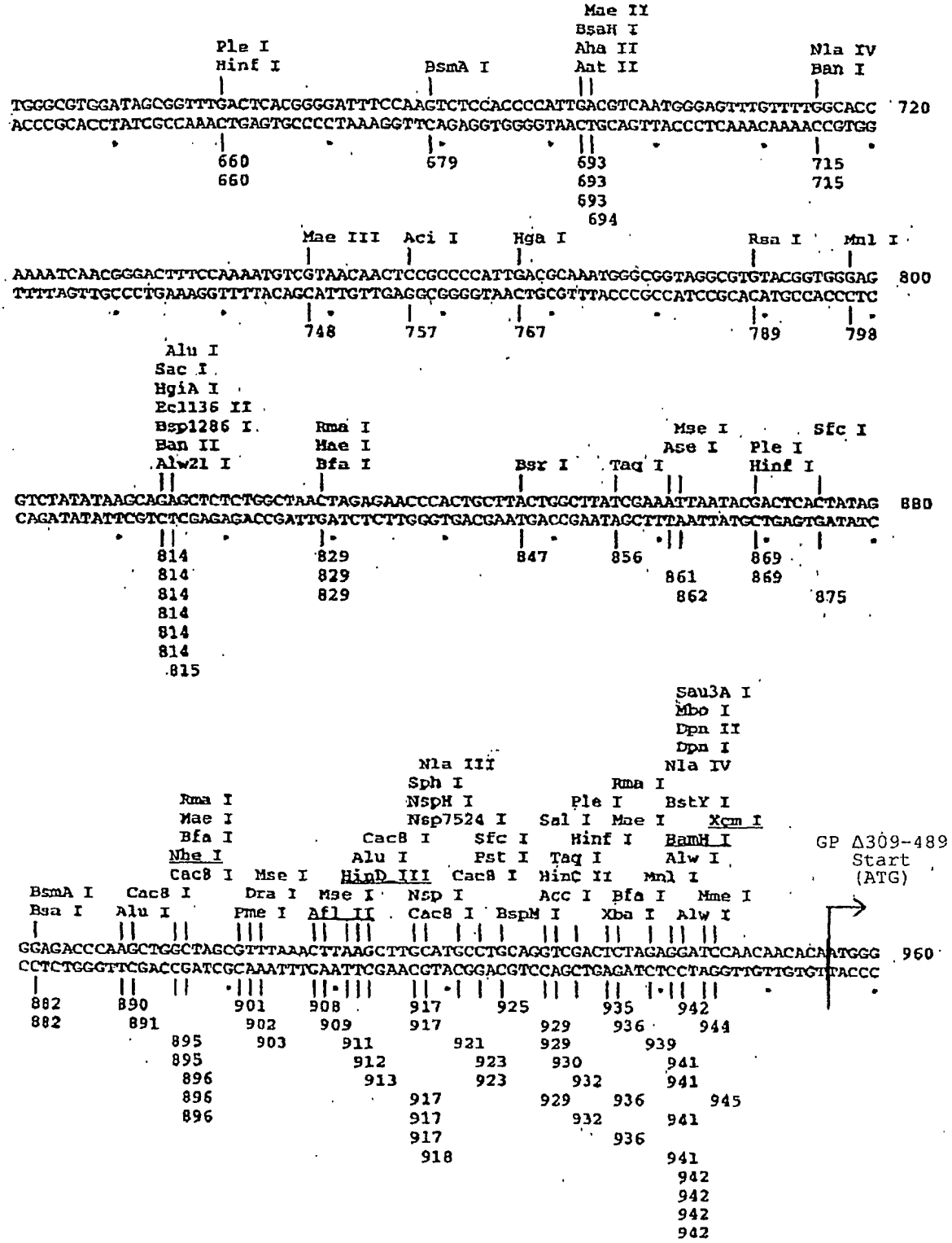
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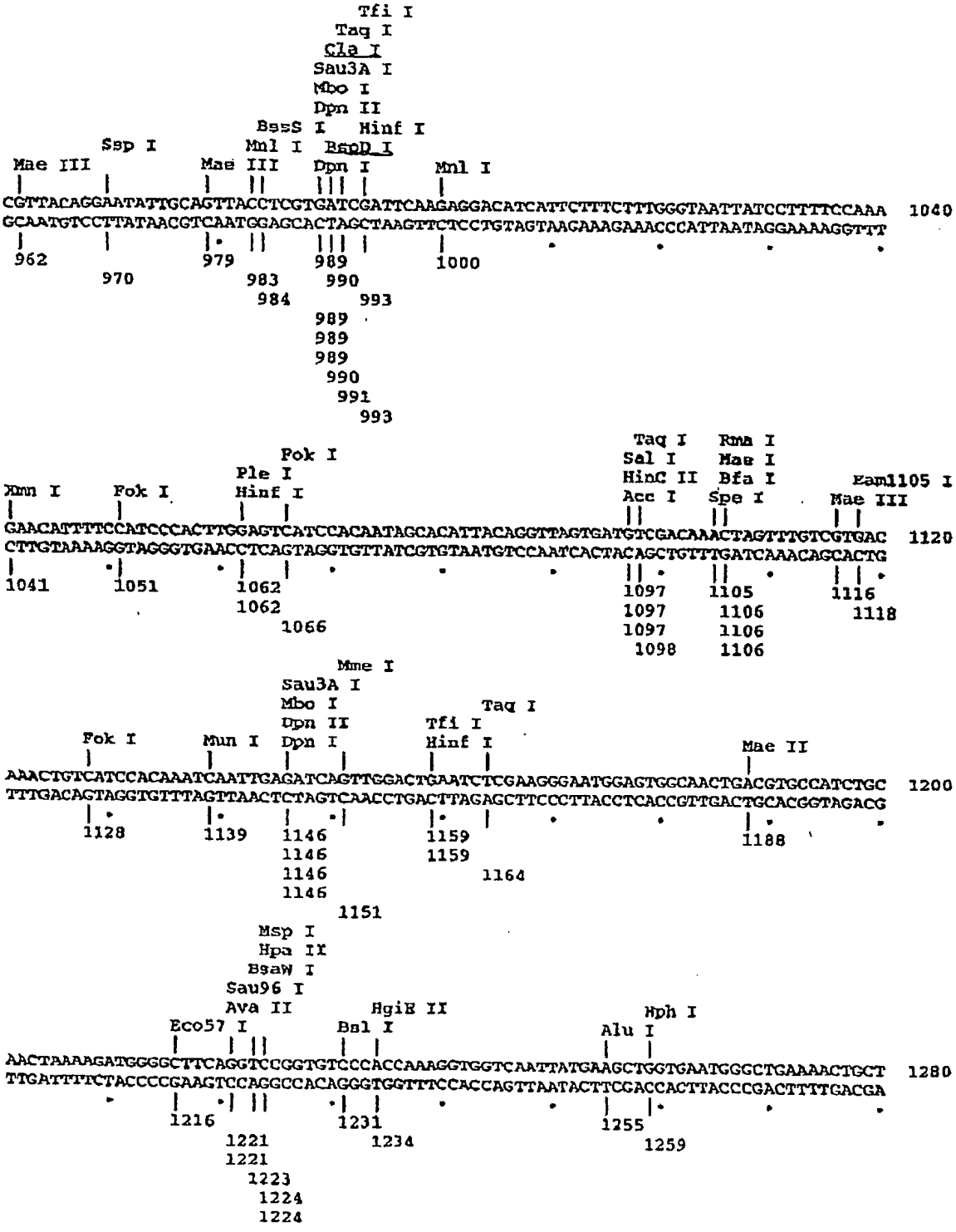
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Spe I
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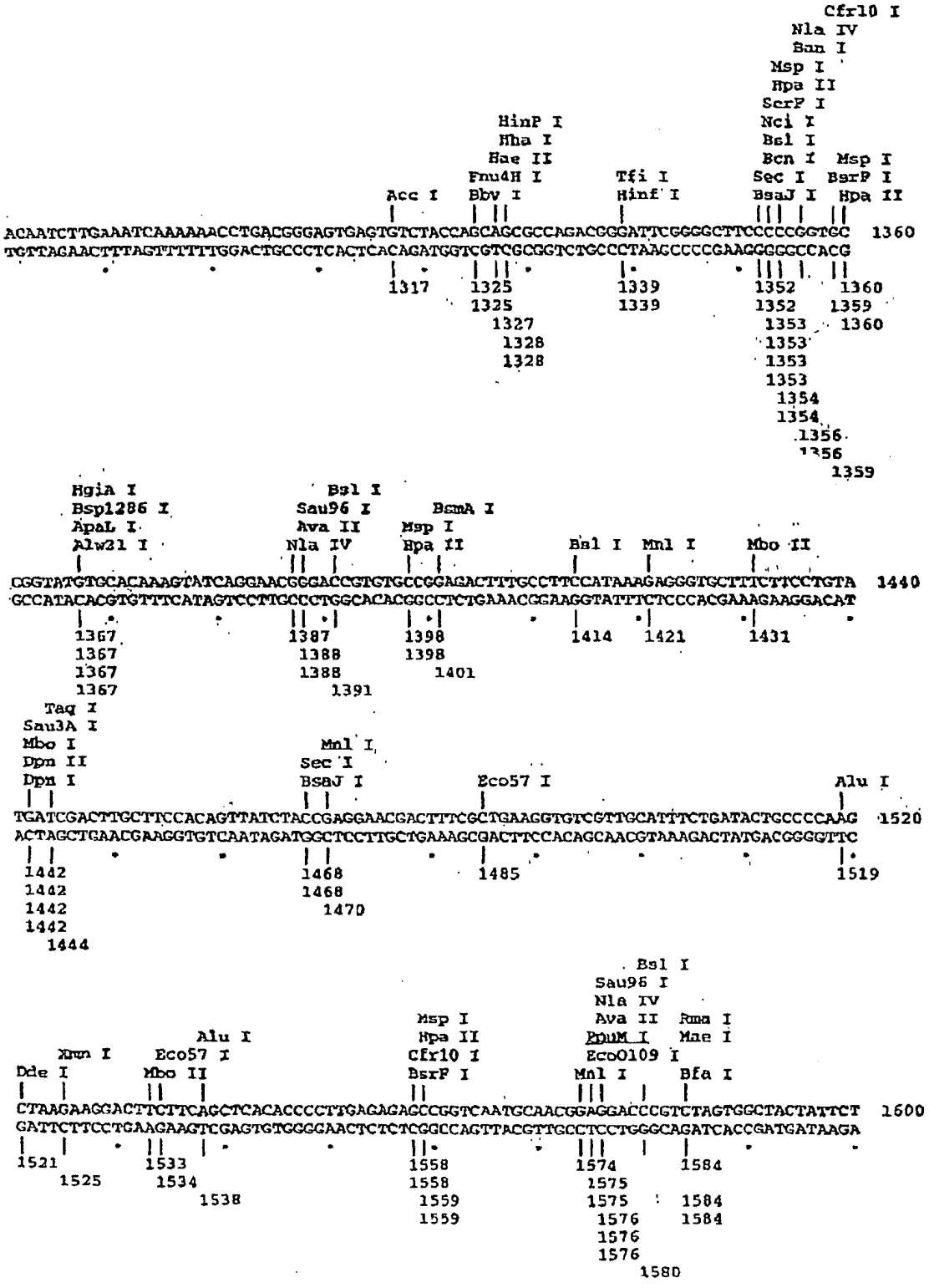
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Mbo I
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Sec I
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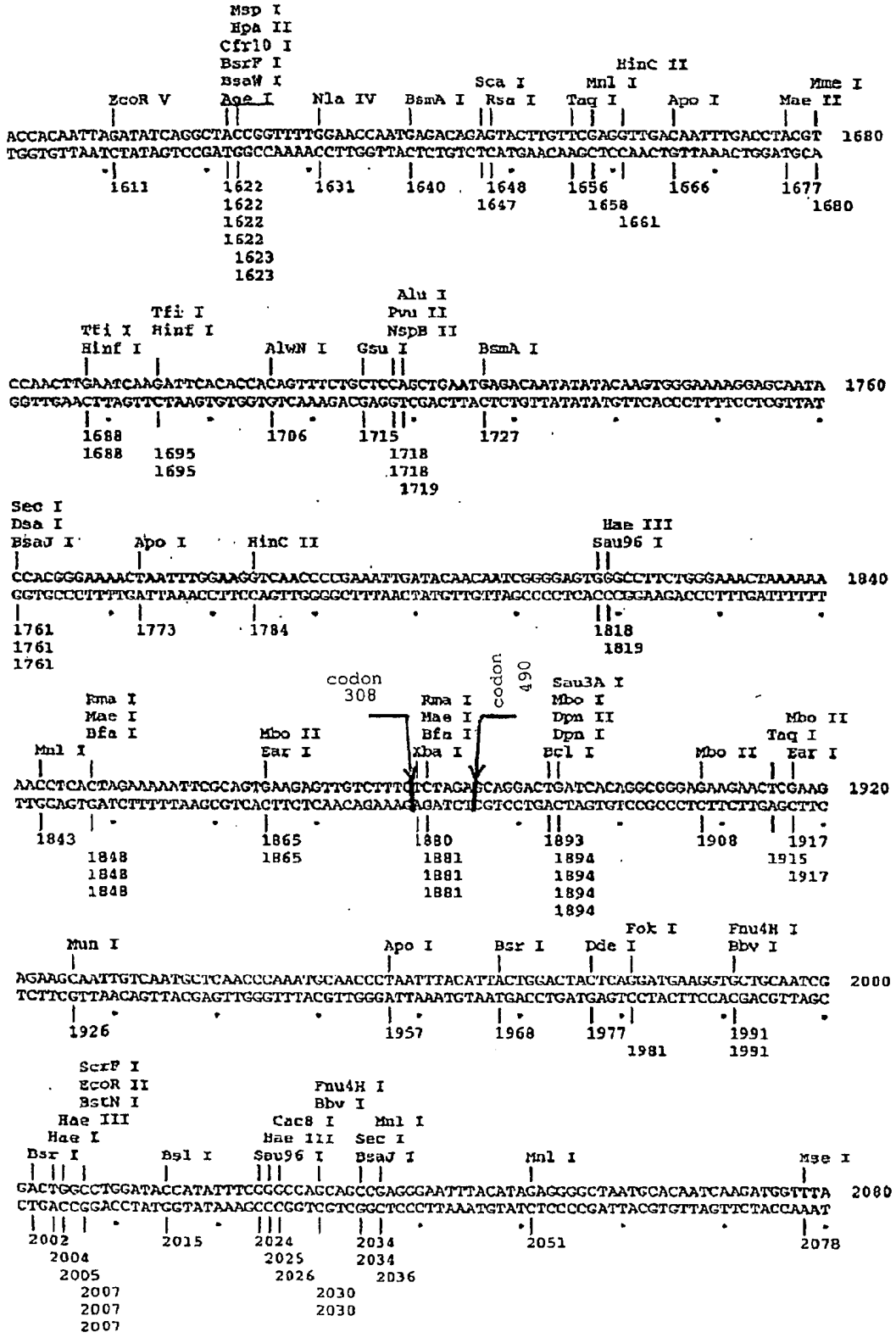
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Apo I
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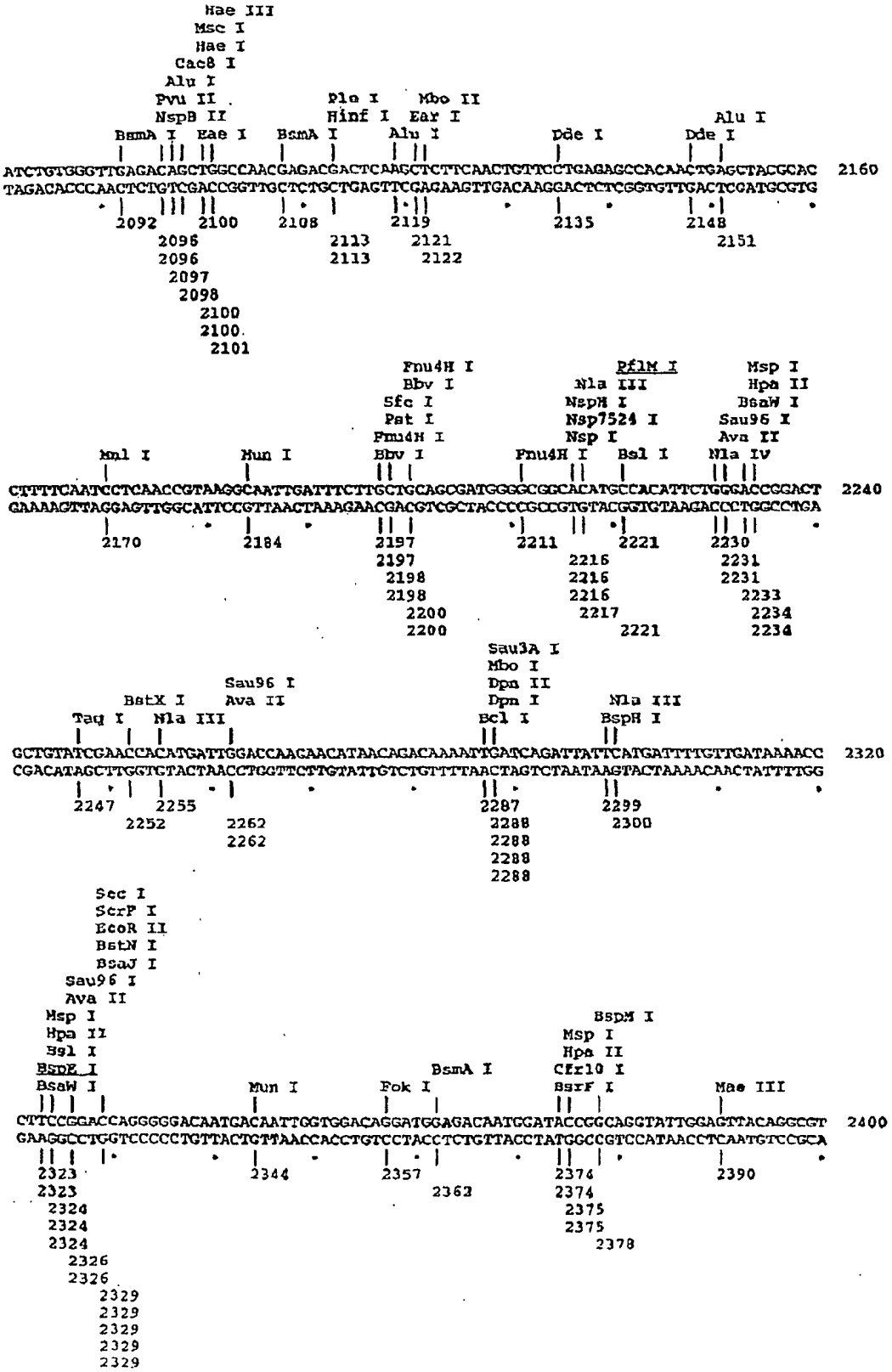
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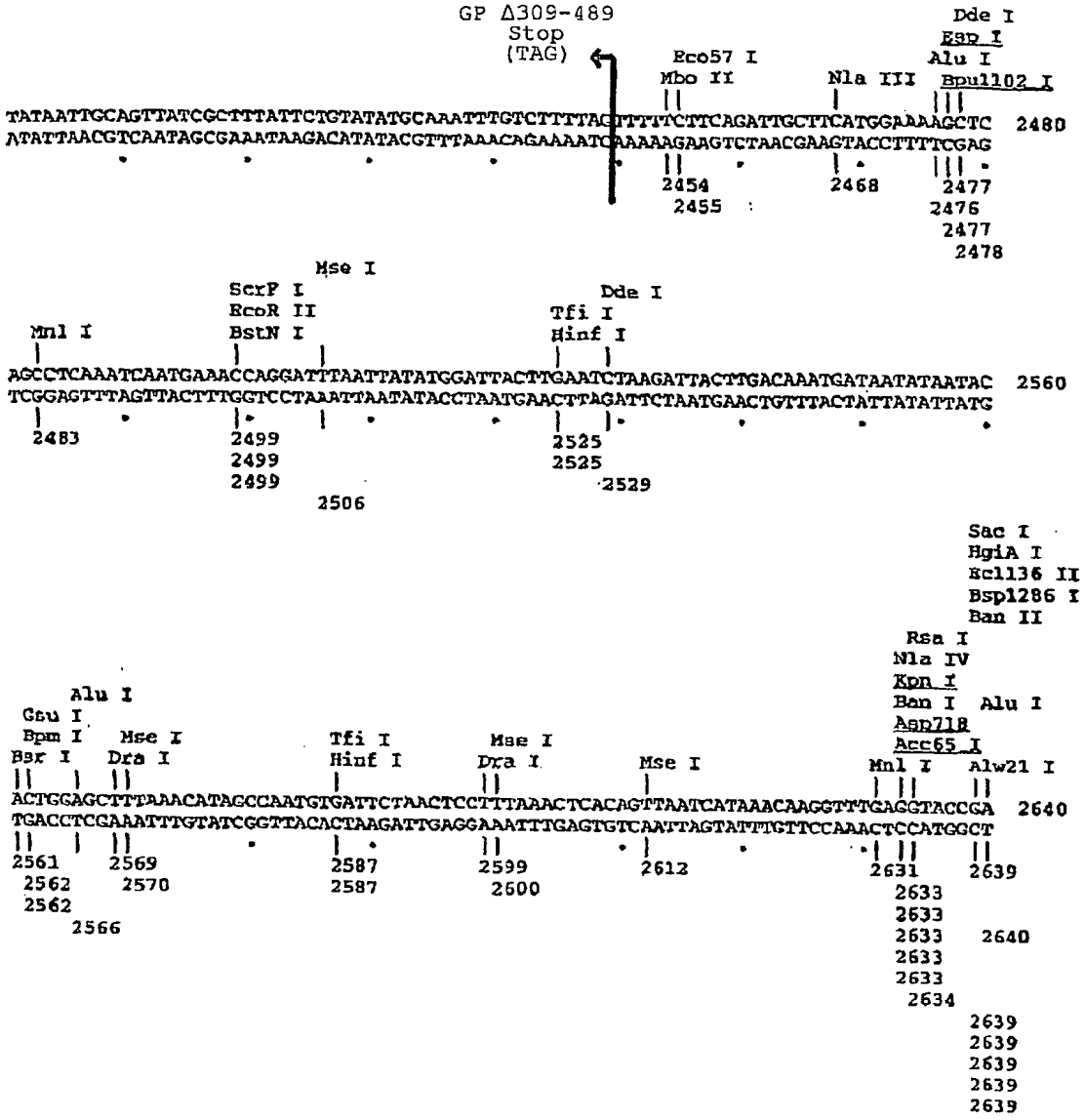
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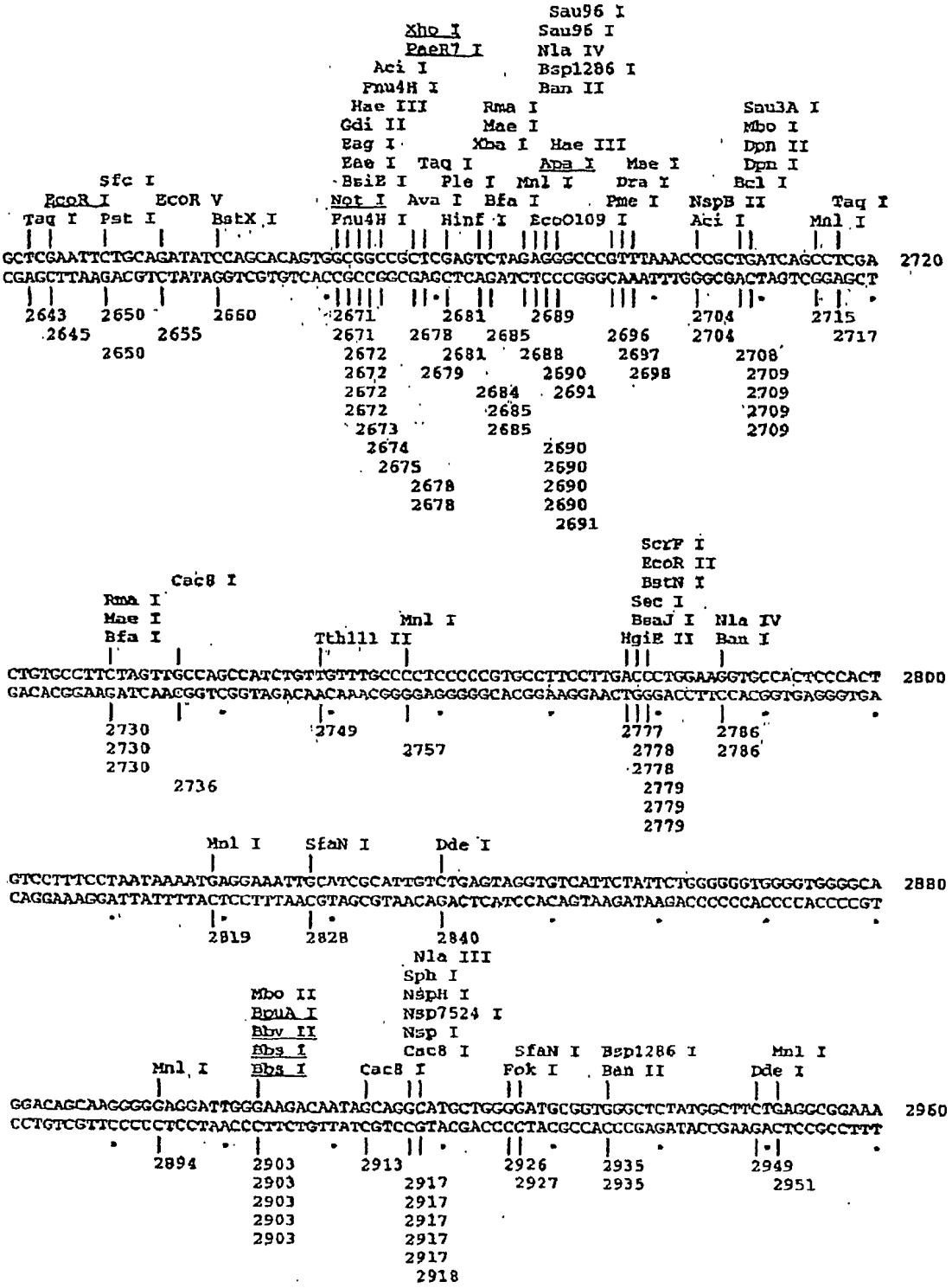
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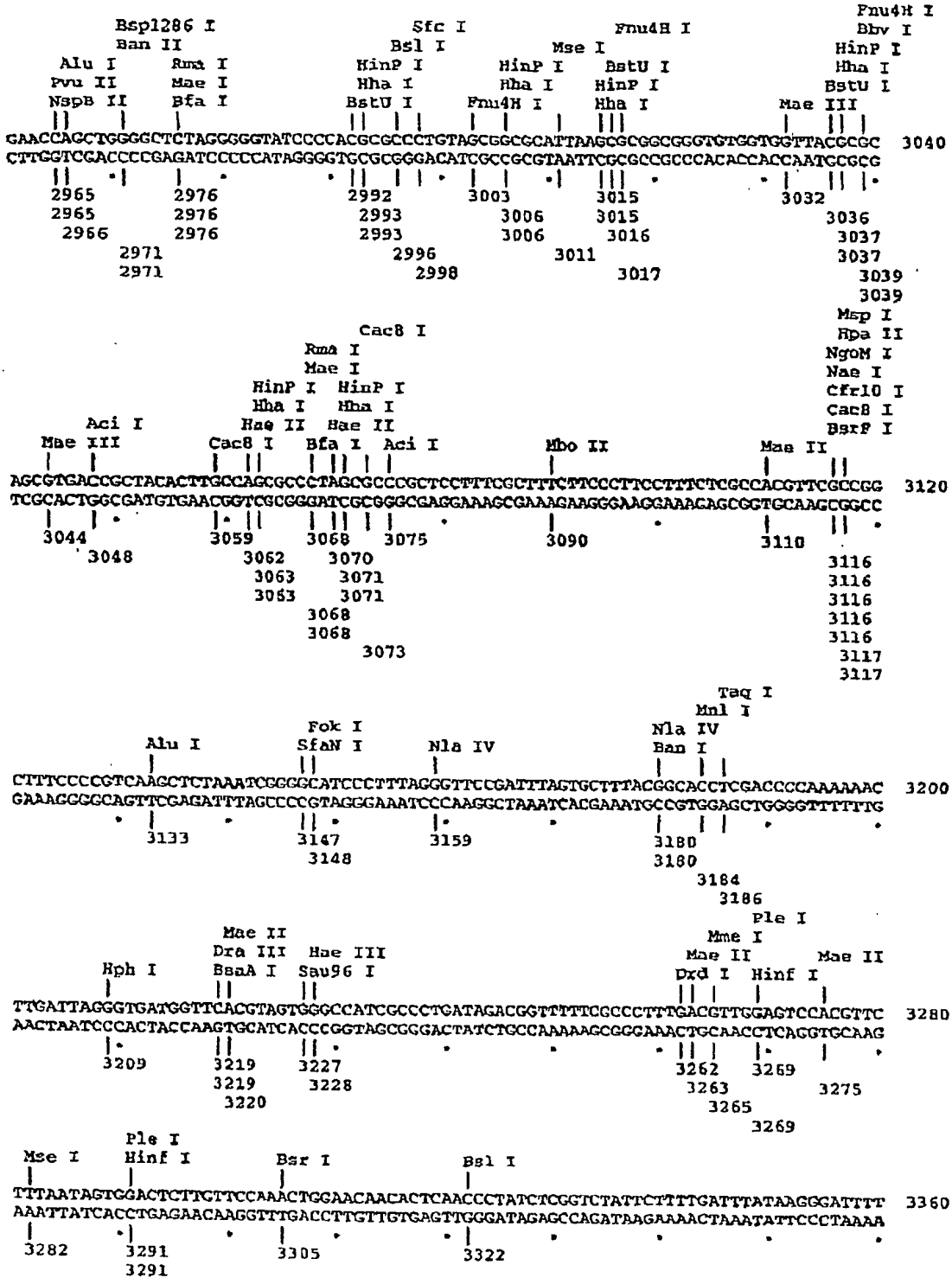
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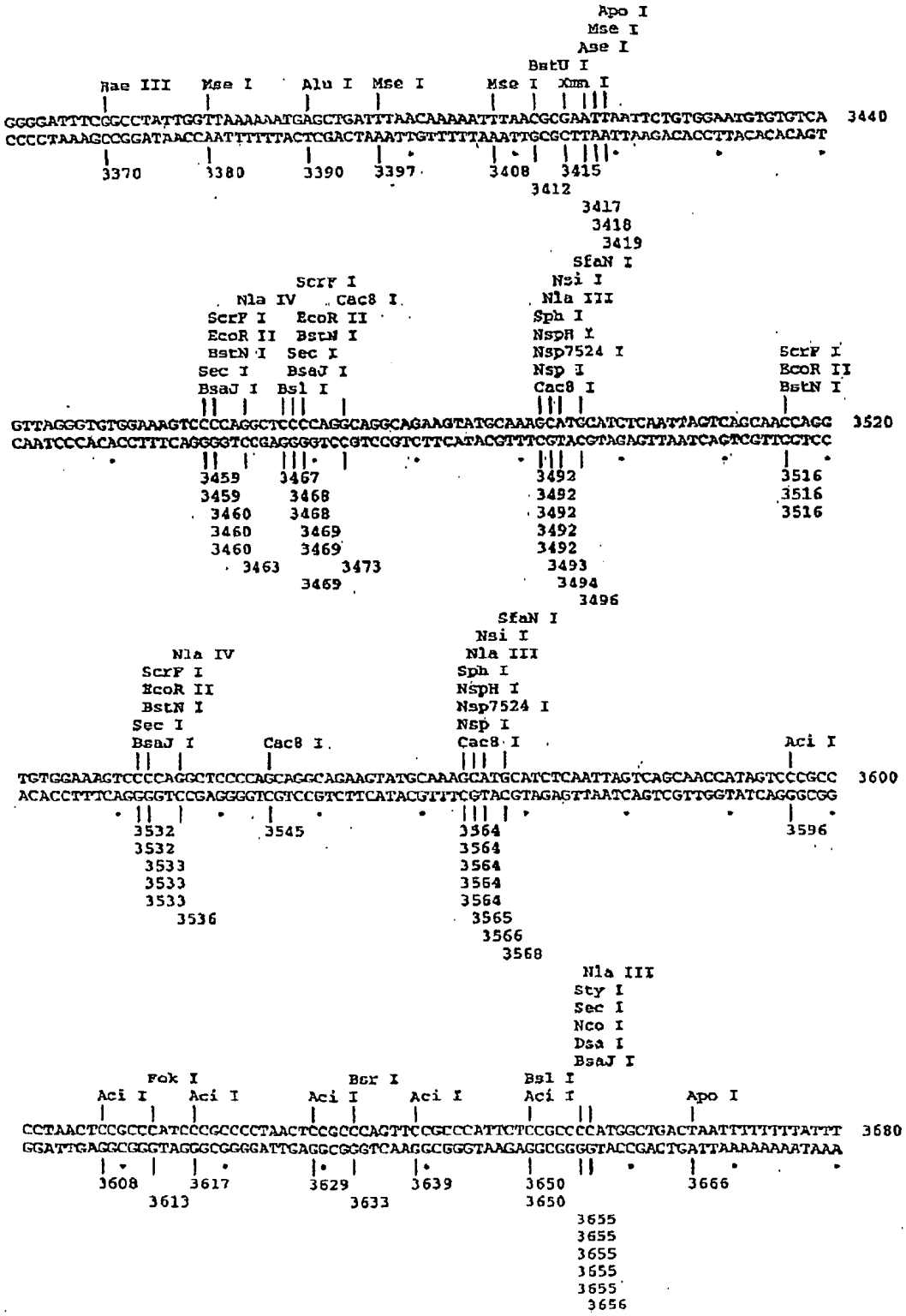
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Sph I
NspH I
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Nsp I
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BstN I
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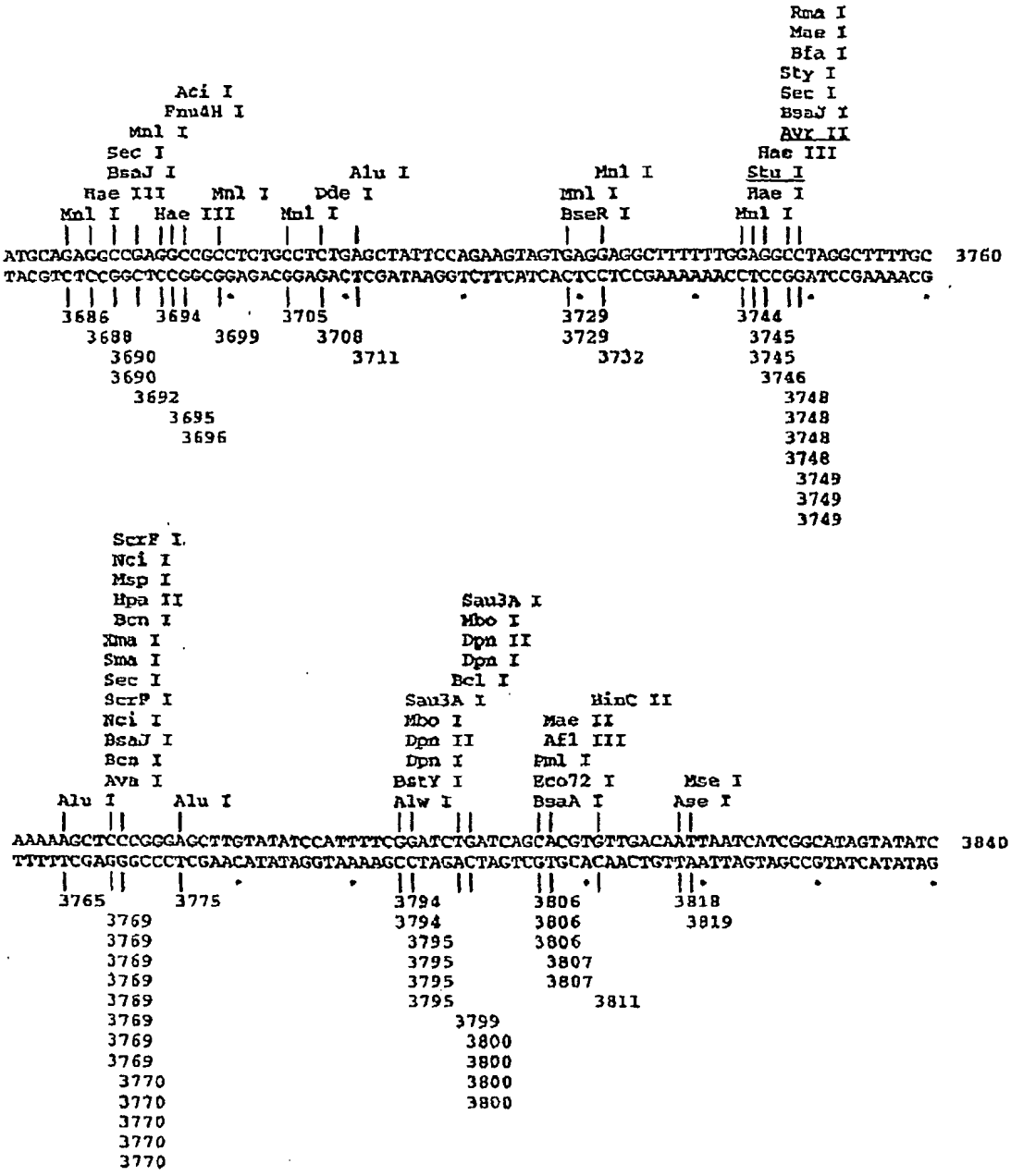
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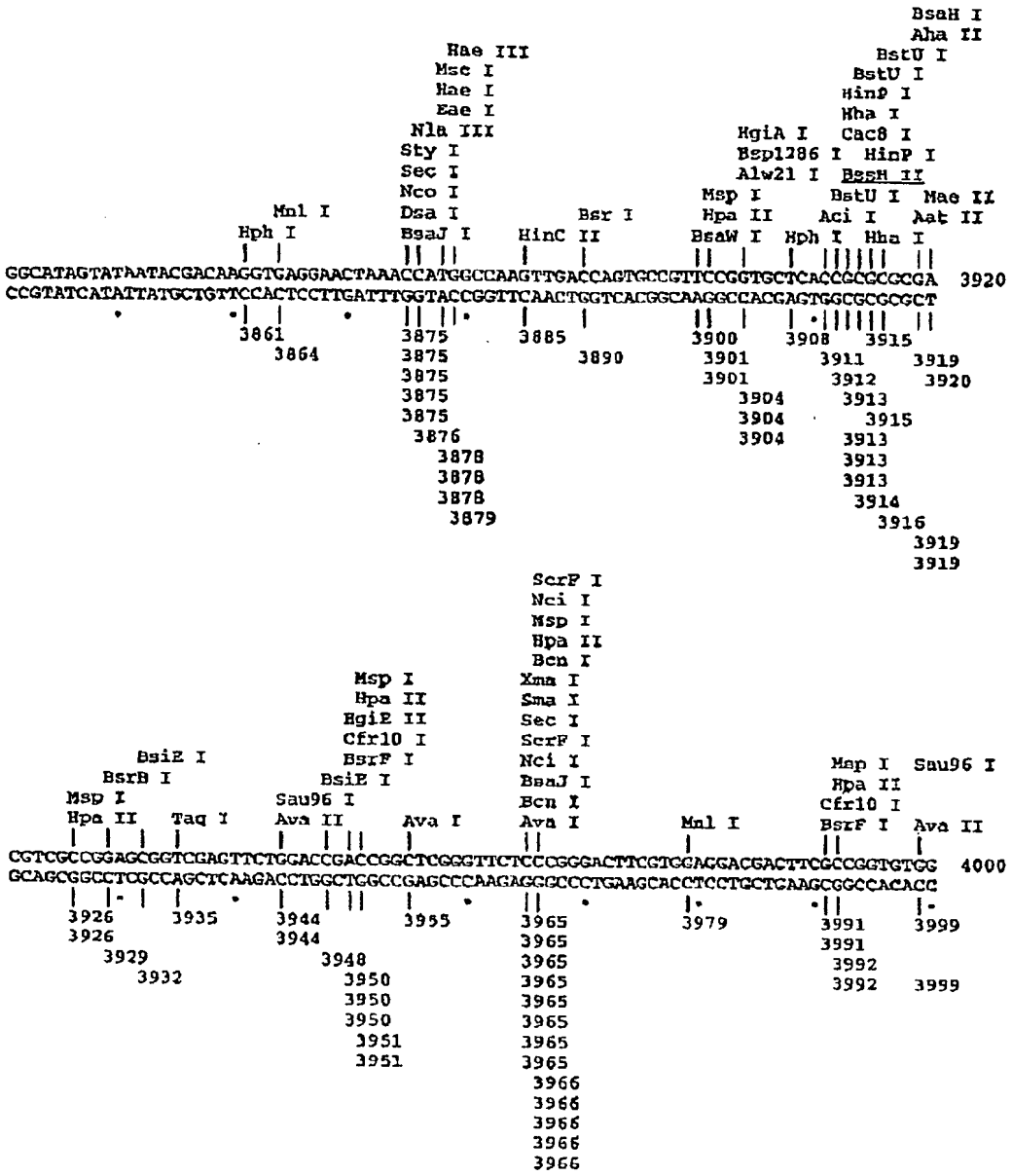
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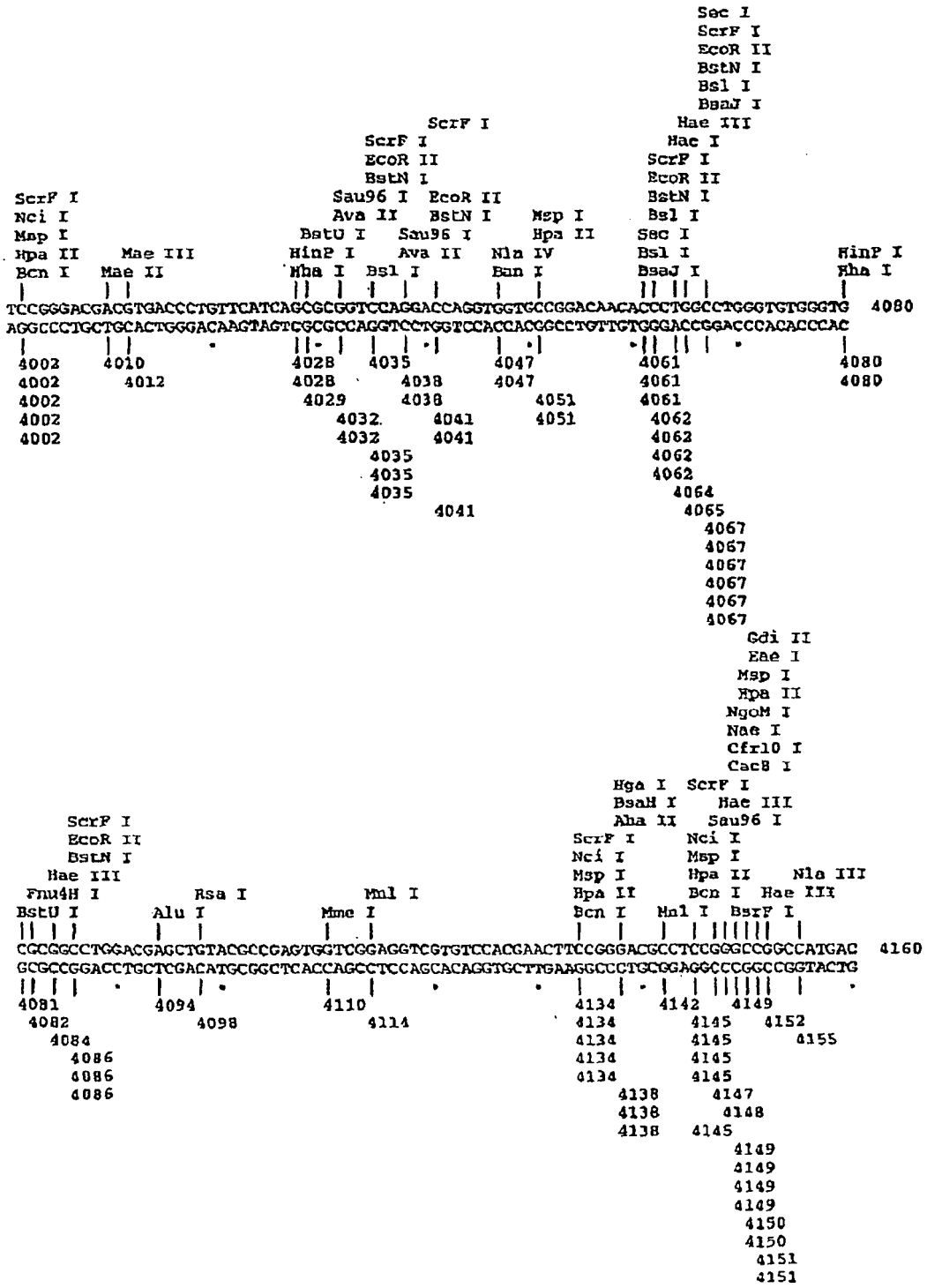
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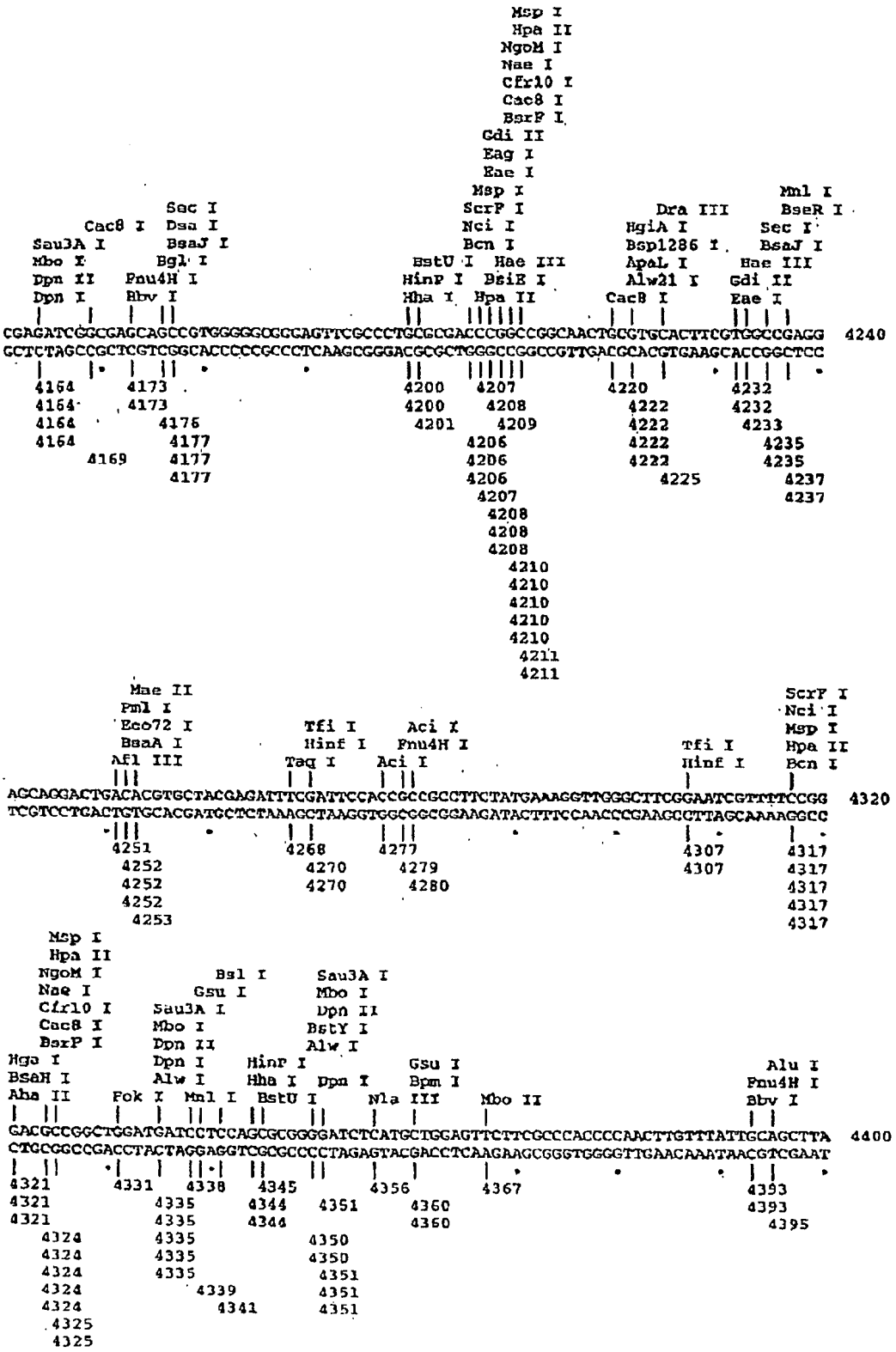
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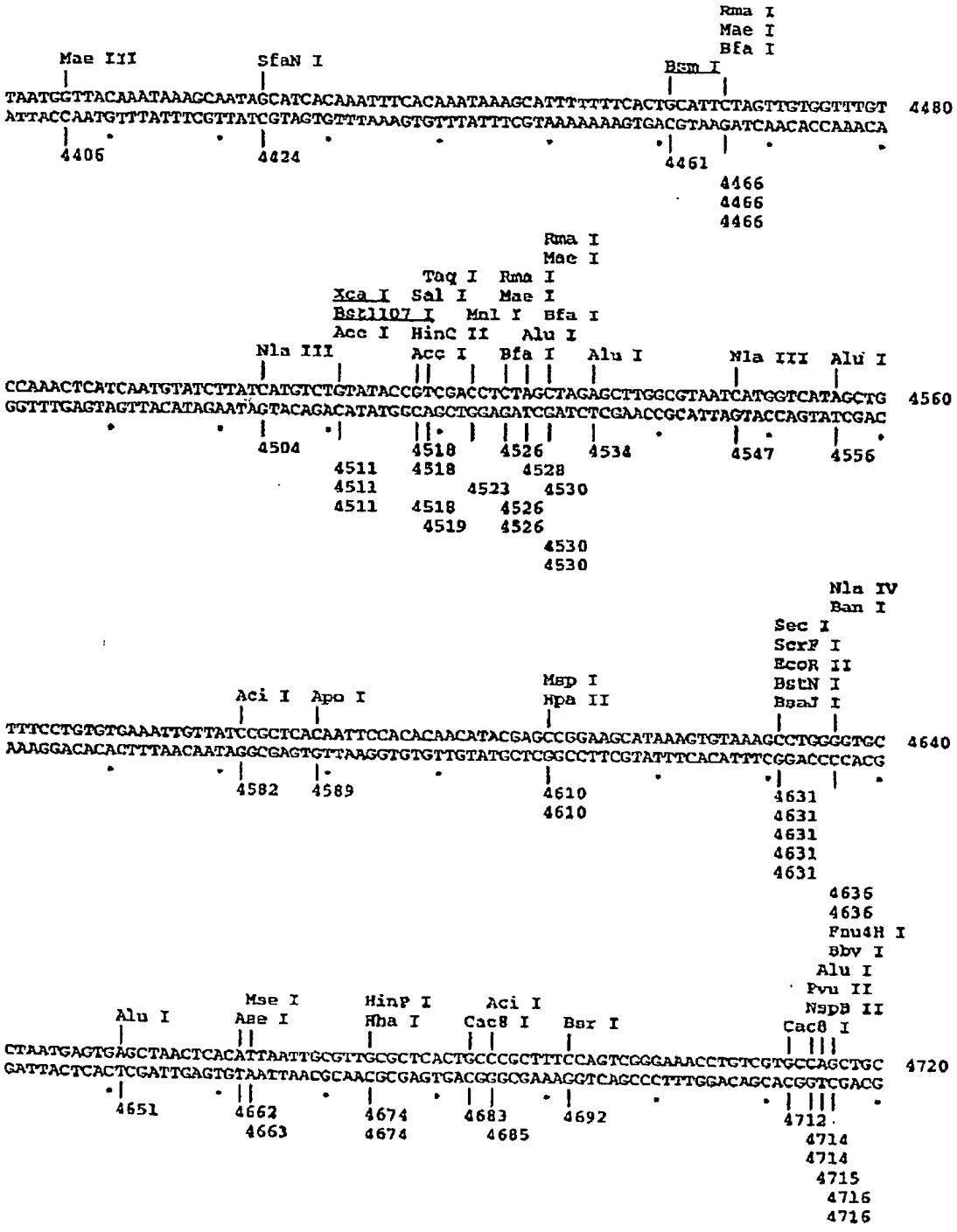
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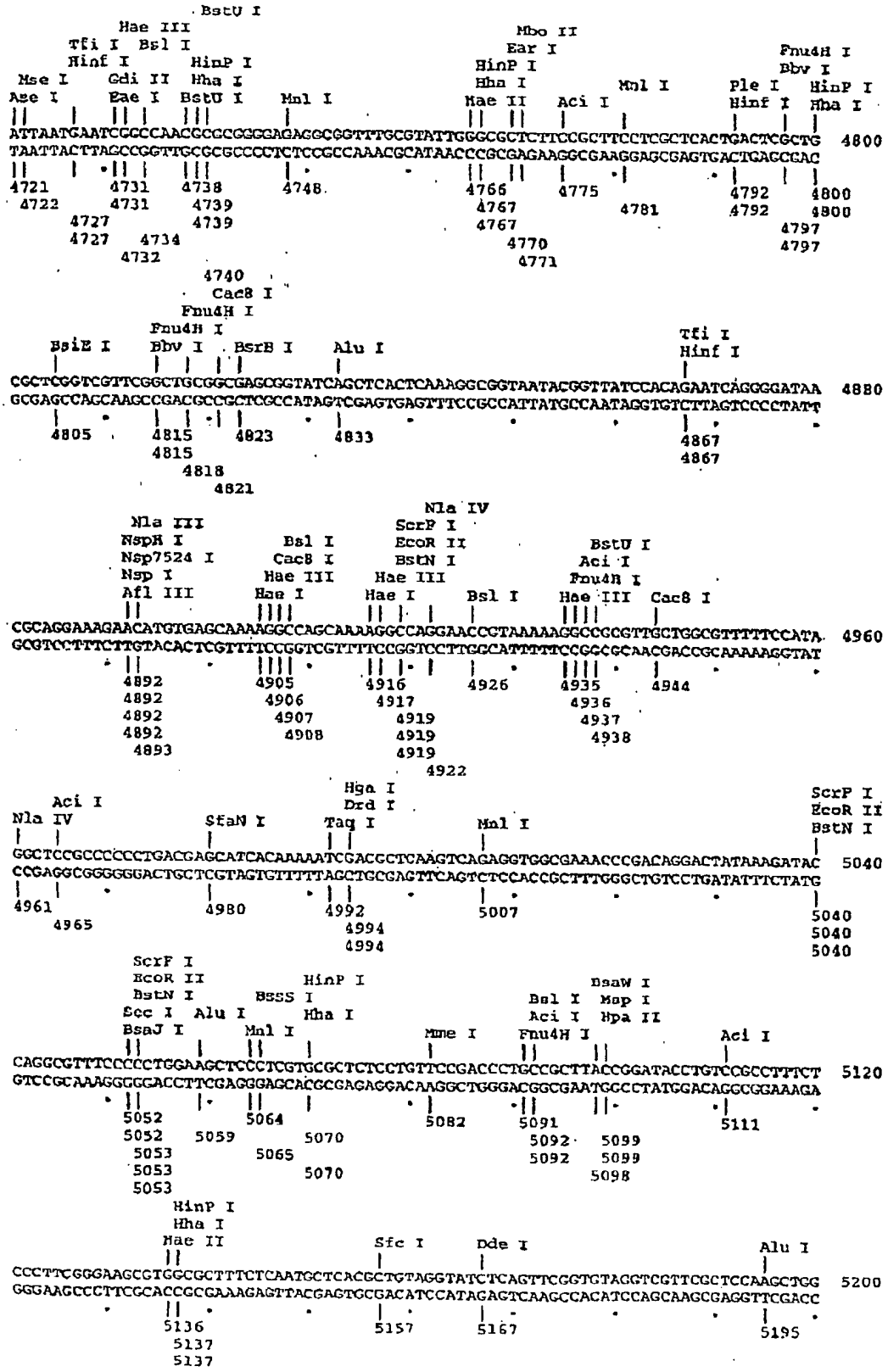
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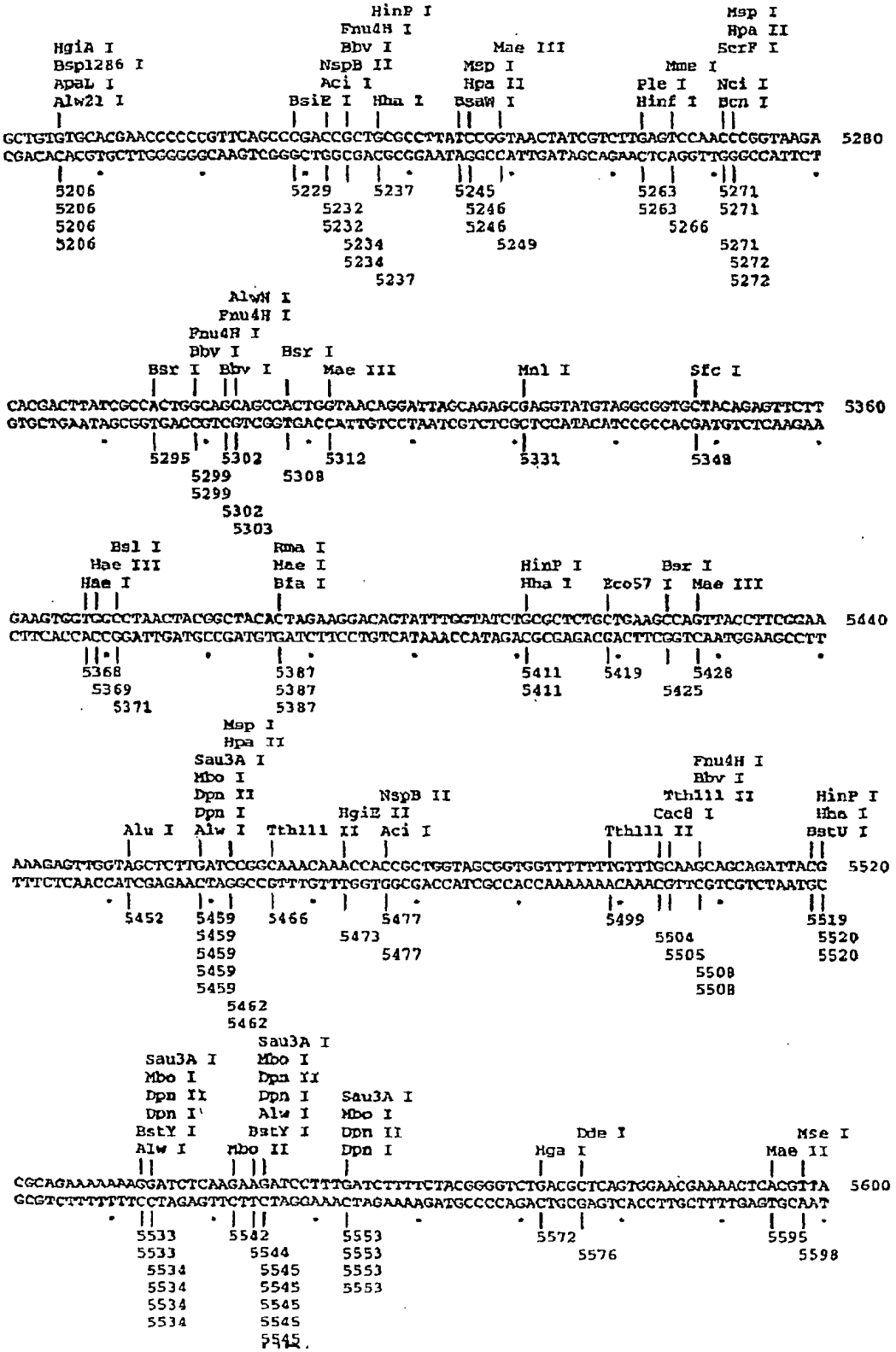
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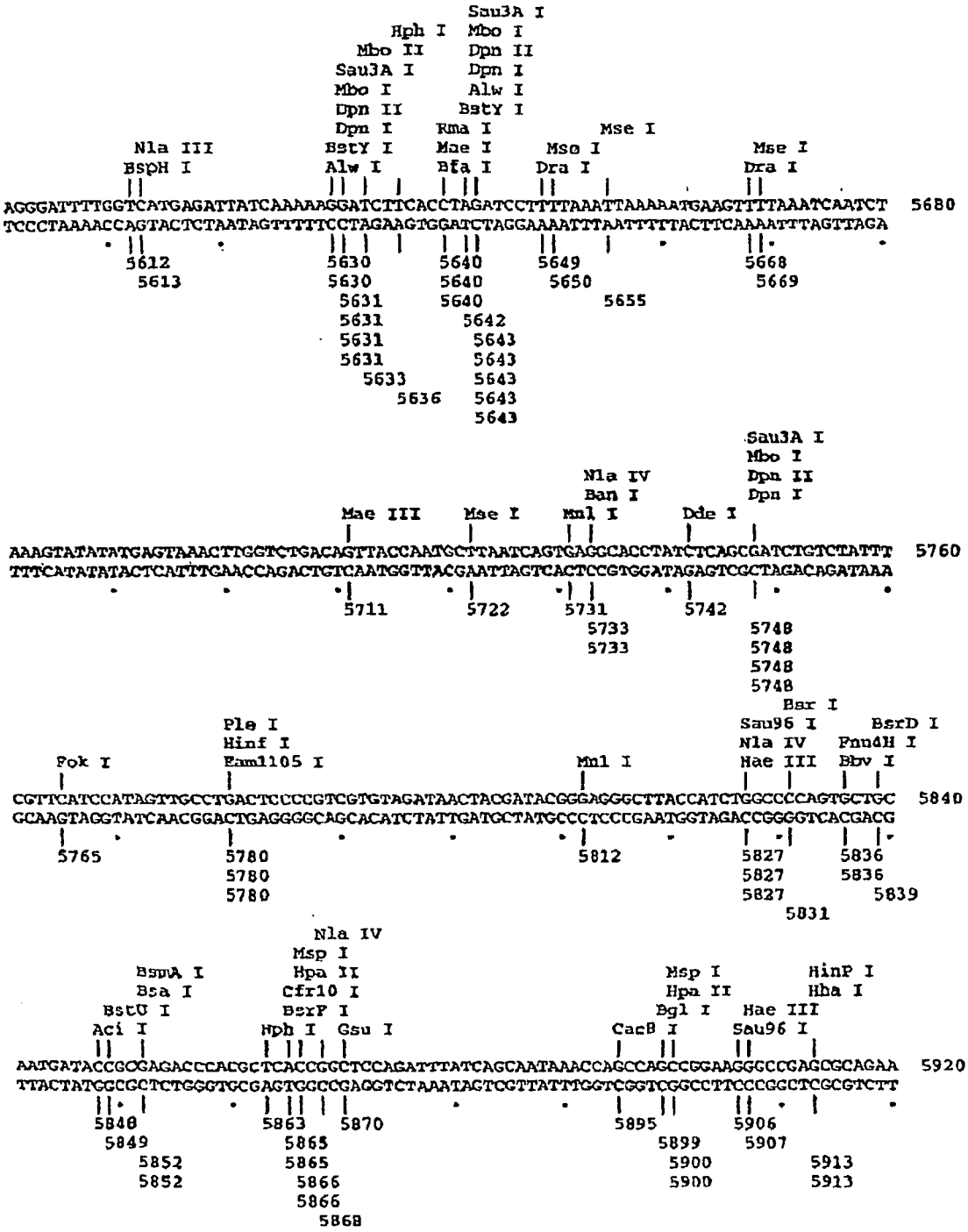
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Bsr I
Ple I
Sau96 I
BsrD I
Hinf I
Nla IV
Fnu4H I
Fok I
Eam1105 I
Mnl I
Hae III
Bbv I
CGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGC 5840
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Nla IV
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BsmA I
Hpa II
Msp I
HinP I
Bsa I
Cfr10 I
Hpa II
Hha I
BstU I
BsrF I
Bgl I
Hae III
Aci I
Nph I
Gsu I
CacB I
Sau96 I
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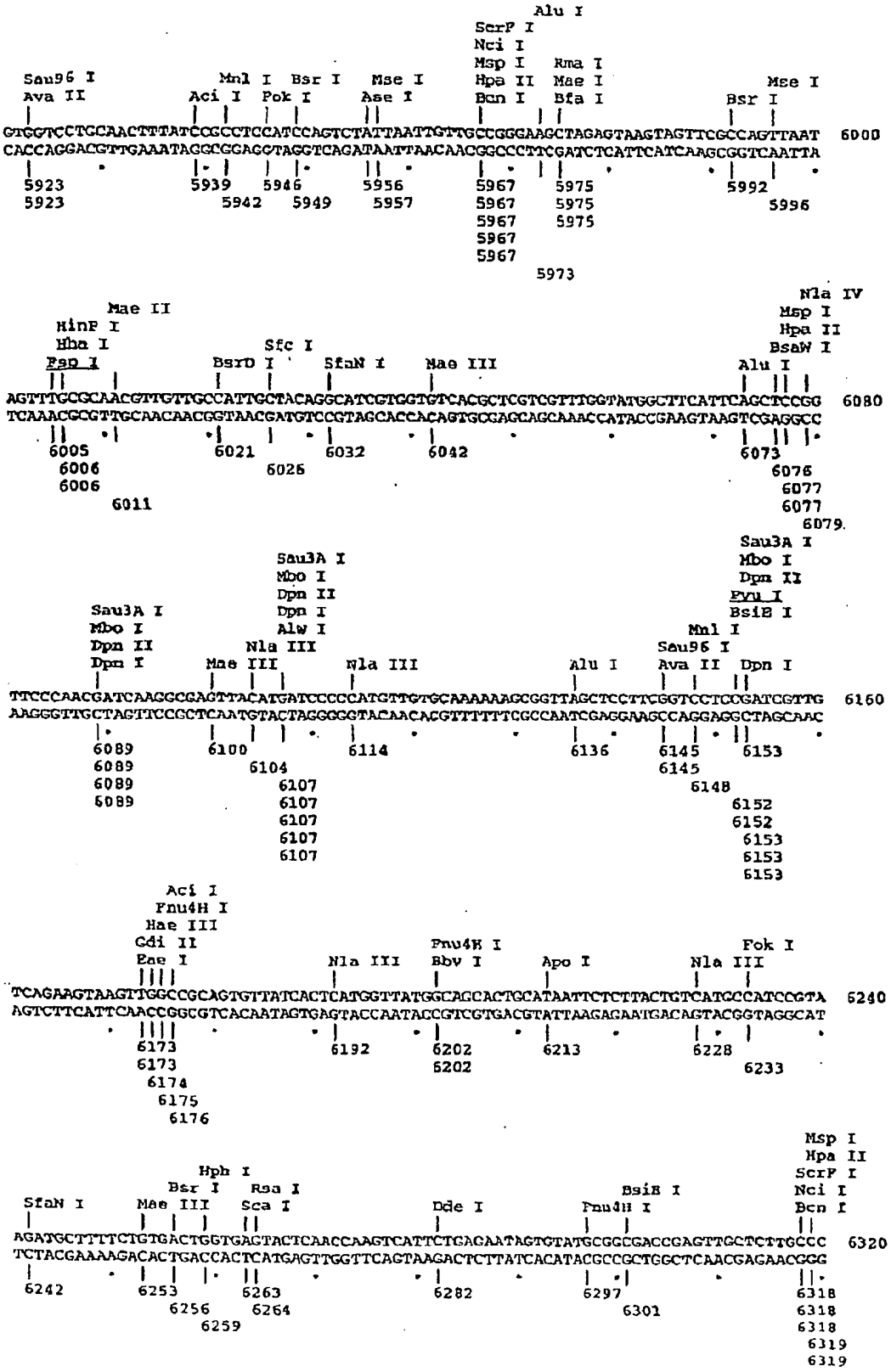
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Bsr I
Pok I
Mse I
Ase I
Bsr I
Mse I
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Hpa II
Hha I
BsaW I
Sfc I
Fsp I
BsrD I
SfaN I
Mae III
Alu I
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Dpn II
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Mbo I
Dpn II
Dpn I
Alw I
Pvu I
BsiE I
Sau3A I
Mbo I
Dpn II
Dpn I
Nla III
Mnl I
Sau96 I
Mae III
Nla III
Alu I
Ava II
Dpn I
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Hae III
Cdi II
Pae I
Fnu4K I
Nla III
Bbv I
Apo I
Fok I
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ScrF I
Bsr I
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SfaN I
Mae III
Sca I
Dde I
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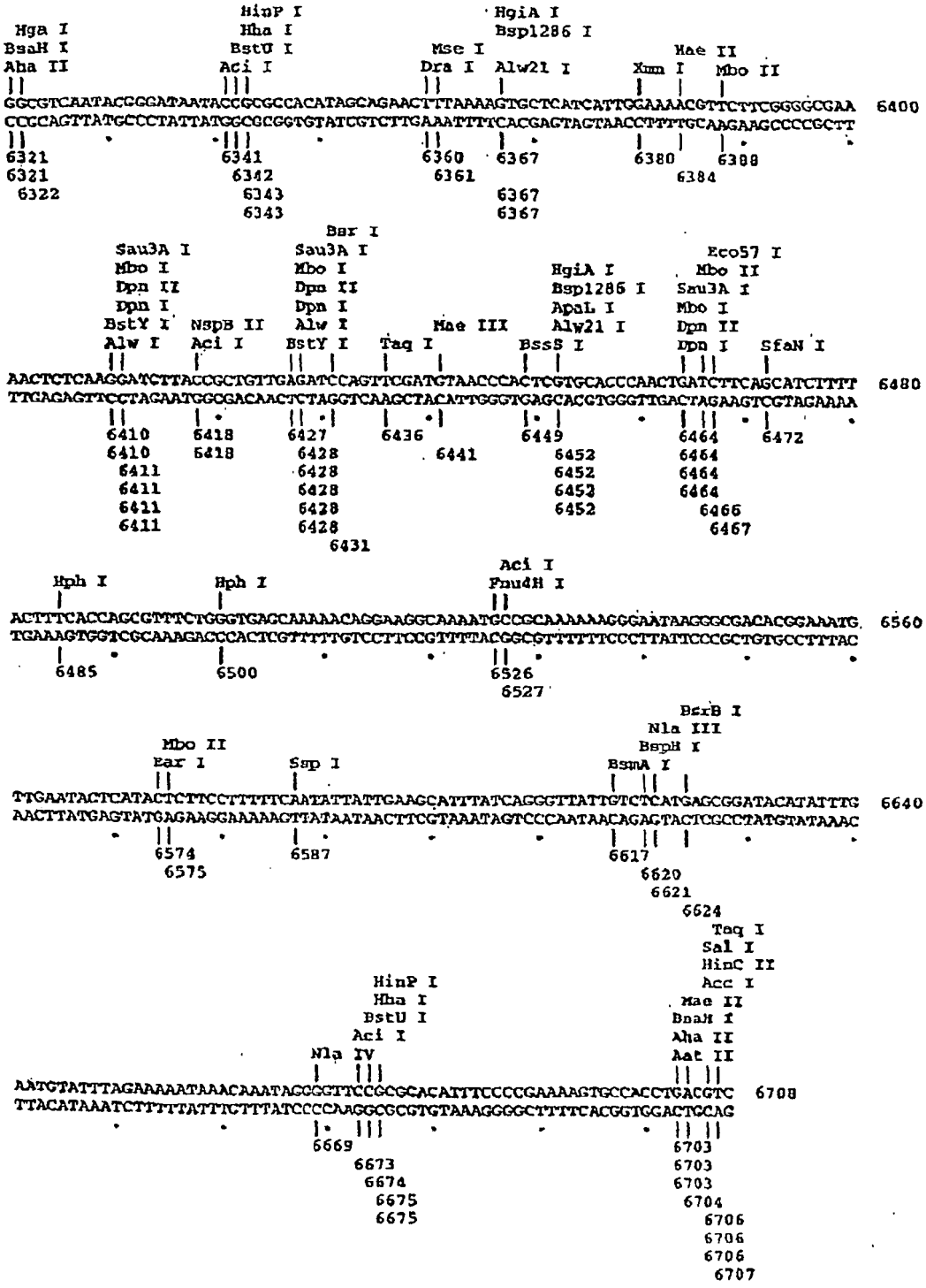

Restriction Endonucleases site usage

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Acc I	7	BsmA I	10	Fok I	12	NspB II	8
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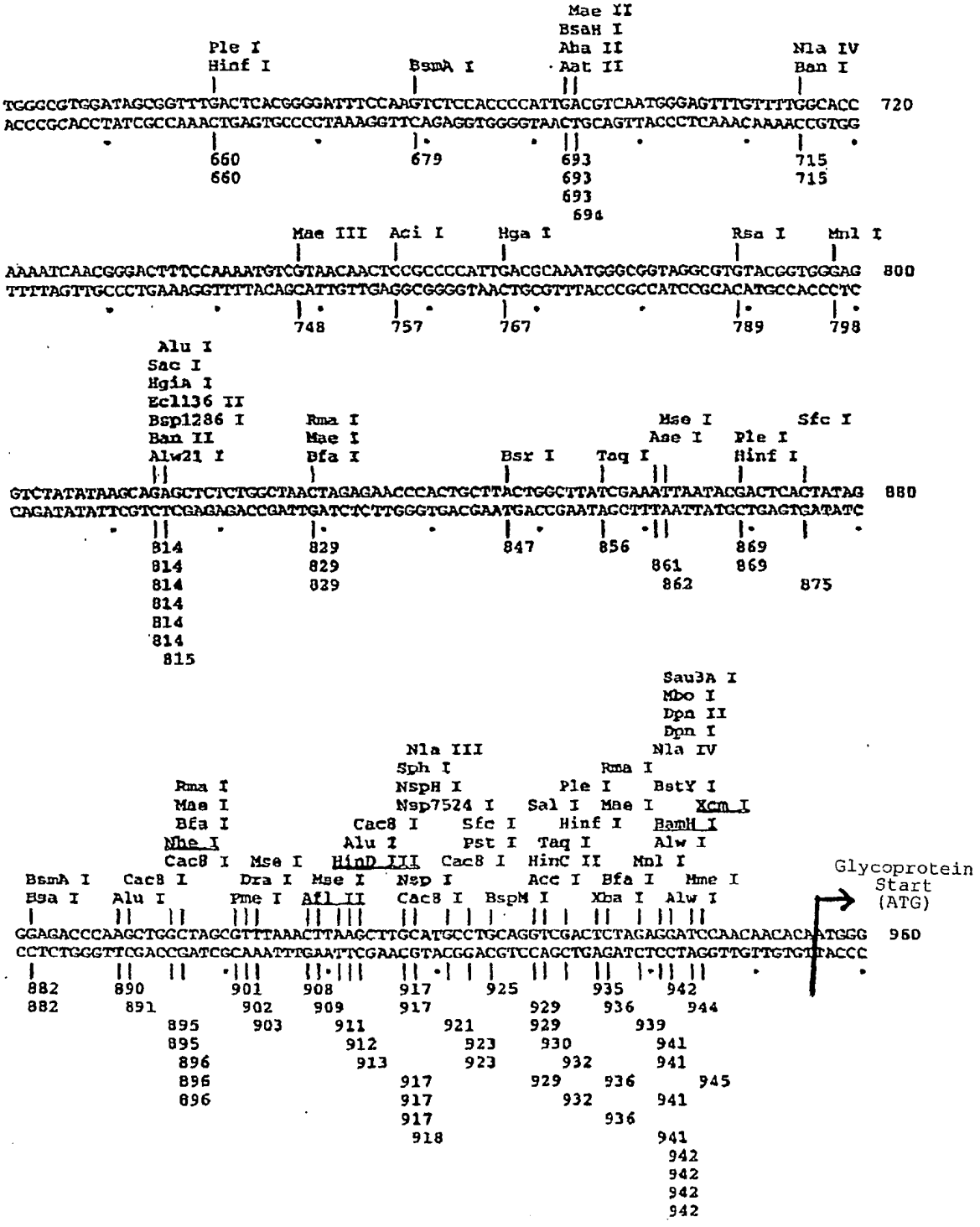
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660 693 715
693
694
Mae III
Aci I
Hga I
Rsa I
Mnl I
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Alu I
Sac I
HgiA I
Ecl136 II
Bsp1286 I
Ban II
Alw21 I
Rma I
Mae I
Bfa I
Bsr I
Taq I
Mse I
Ase I
Ple I
Hinf I
Sfc I
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814 829
814
814
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815
Sau3A I
Mbo I
Dpn II
Dpn I
Nla III
Nla IV
Sph I
Rma I
NspH I
Ple I
BstY I
Rma I
Mae I
Nsp7524 I
Sal I
Mae I
Xcm I
Bfa I
Cac8 I
Sfc I
Hinf I
BamH I
Nhe I
Alu I
Pst I
Taq I
Alw I
Cac8 I
Mse I
HinD III
Cac8 I
HinC II
Mnl I
BsmA I
Cac8 I
Dra I
Mse I
Nsp I
Acc I
Bfa I
Mme I
Bga I
Alu I
Pme I
Afl II
Cac8 I
BspM I
Xba I
Alw I
Glycoprotein
Start
(ATG)
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CCTCTGGGTTCGACCGATCGCAAATTTGAATTCGAACGTACGGACGTCCAGCTGAGATCTCCTAGGTTGTTGTGTTACCC
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882 891 902 909 917 929 936 944
895 903 911 921 929 939
895 912 923 930 941
896 913 923 932 941
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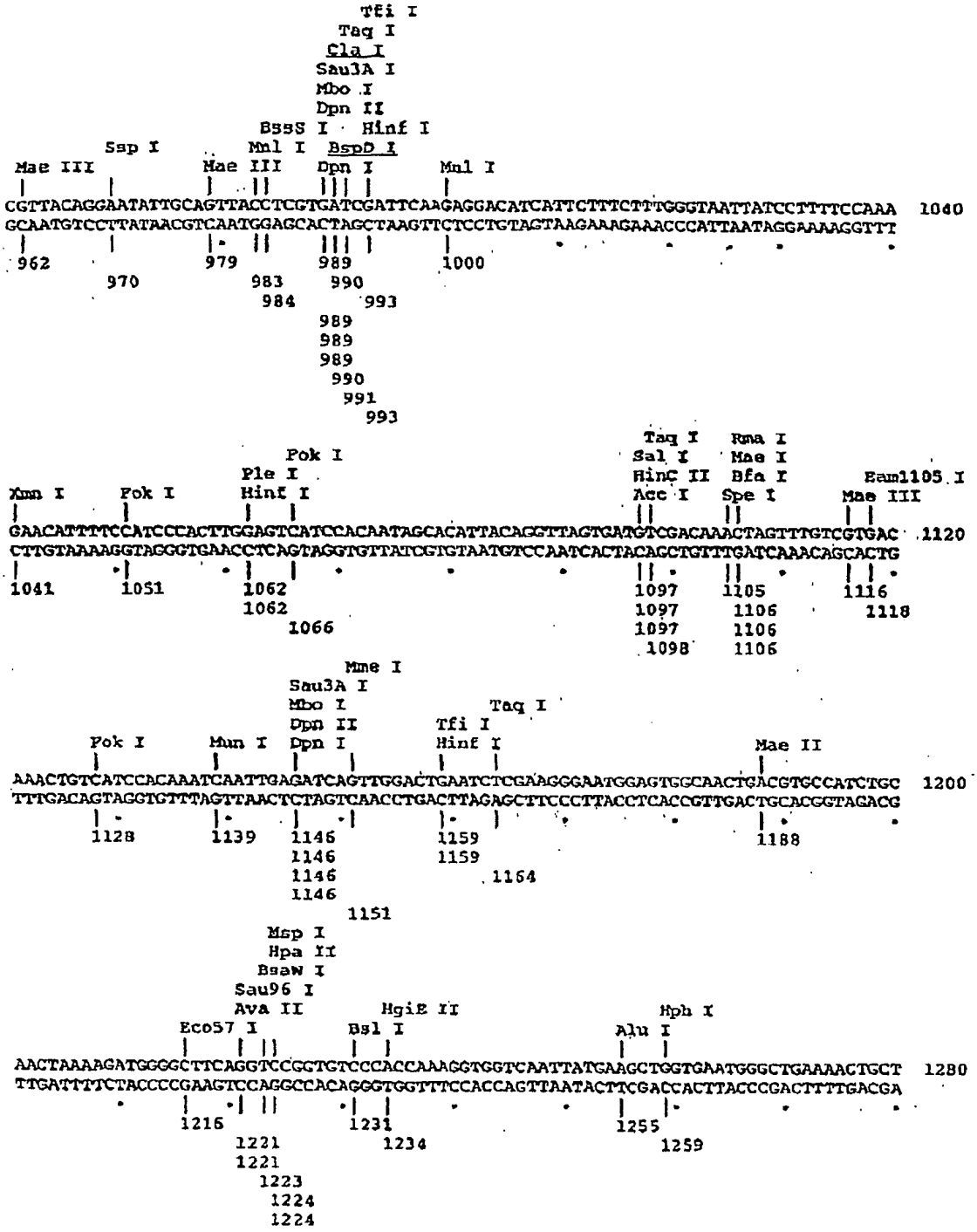
Fig. 1C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Cla I
Sau3A I
Mbo I
Dpn II
BssS I
Hinf I
Ssp I
Mnl I
BspD I
Mae III
Mae III
Dpn I
Mnl I
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Taq I
Rma I
Pok I
Sal I
Mae I
Ple I
Hinc II
Bfa I
Eam1105 I
Xmn I
Pok I
Hinf I
Acc I
Spe I
Mae III
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Sau3A I
Mbo I
Taq I
Dpn II
Tfi I
Pok I
Mun I
Dpn I
Hinf I
Mae II
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BsaW I
Sau96 I
Ava II
HgiE II
Hph I
Eco57 I
Bsl I
Alu I
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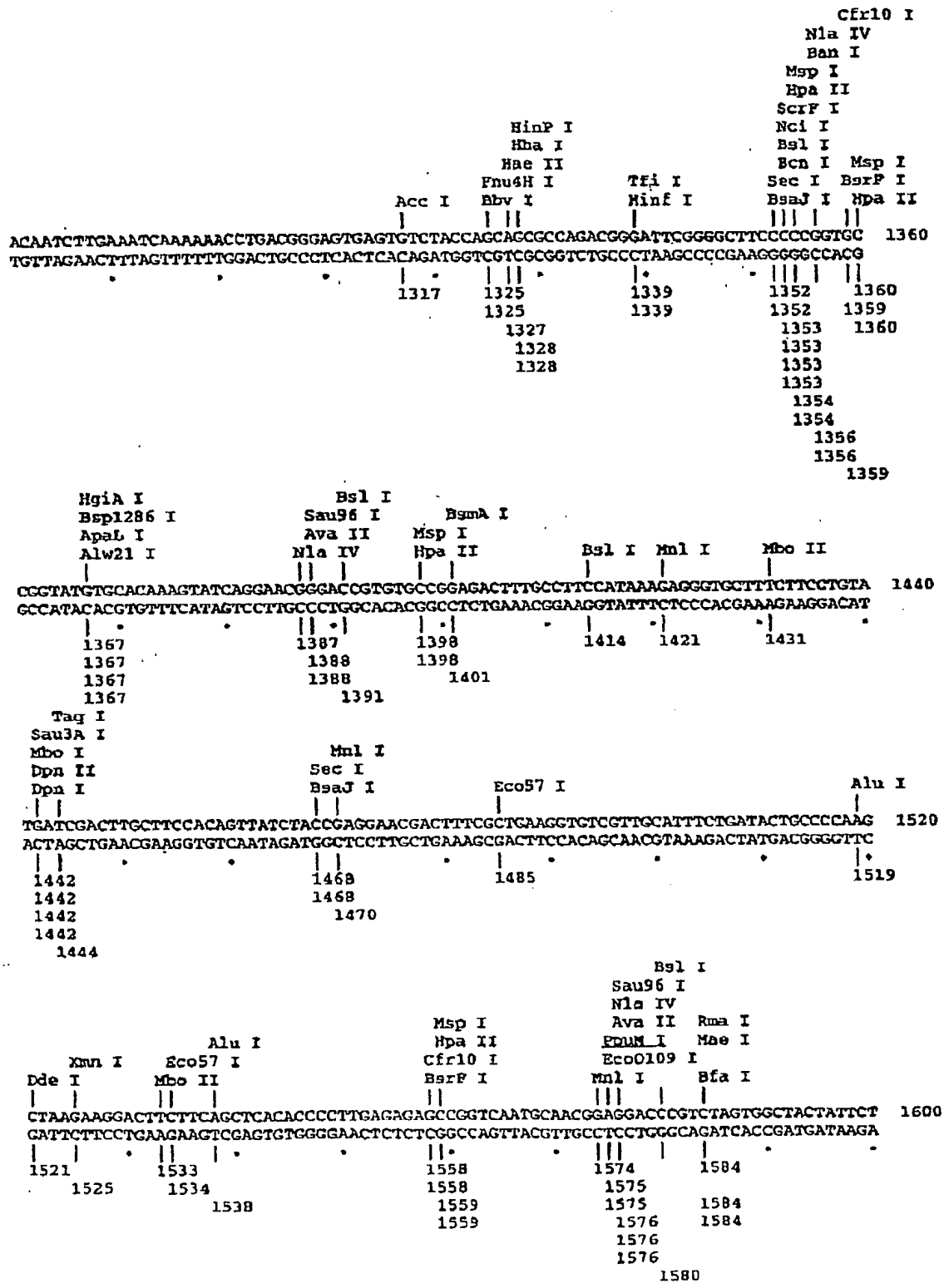
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Msp I
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ScrF I
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HinP I
Hha I
Hae II
Fnu4H I
Acc I Bbv I
Tfi I
Hinf I
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Bsp1286 I
ApaL I
Alw21 I
Bsl I
Sau96 I
Ava II
Nla IV
BsmA I
Msp I
Hpa II
Bsl I Mnl I Mbo II
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Taq I
Sau3A I
Mbo I
Dpn II
Dpn I
Mnl I
Sec I
BsaJ I
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Alu I
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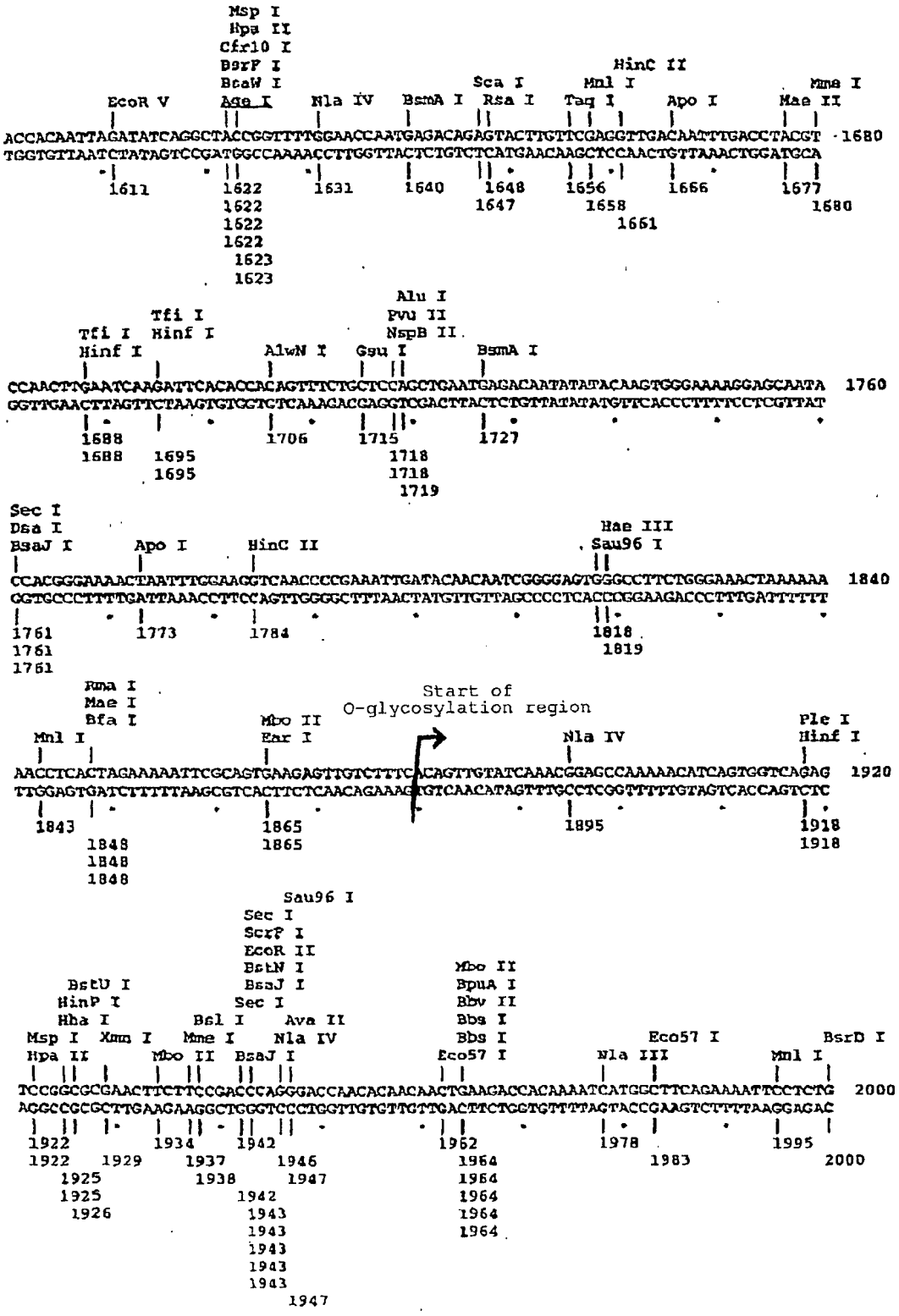
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Mnl I
Taq I
Apo I
Mae II
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BsaJ I
Apo I
HinC II
Hae III
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Bfa I
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Ear I
Start of
O-glycosylation region
Nla IV
Ple I
Hinf I
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Sec I
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Msp I
Hpa II
Xmn I
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BsrD I
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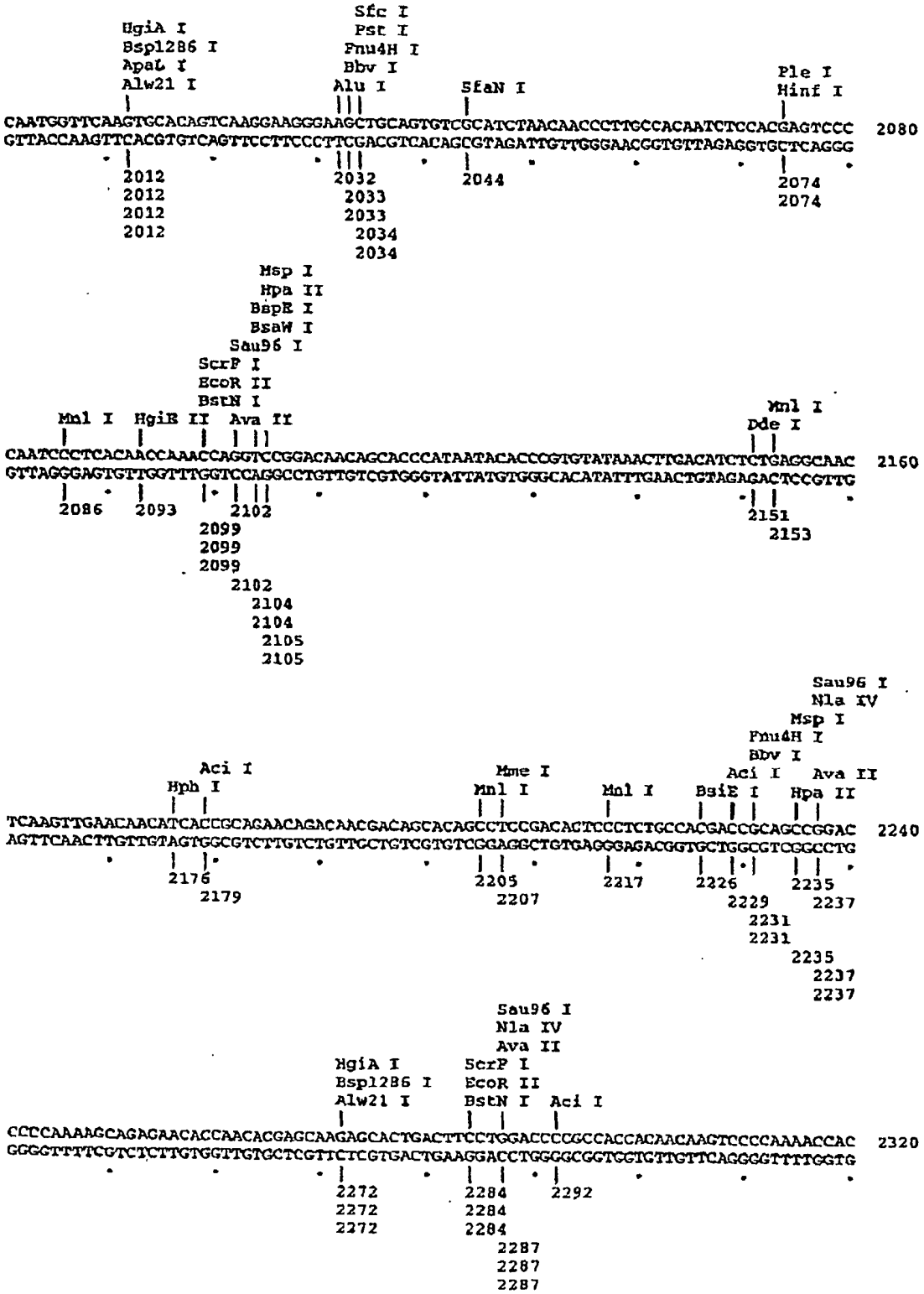
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Hpa II
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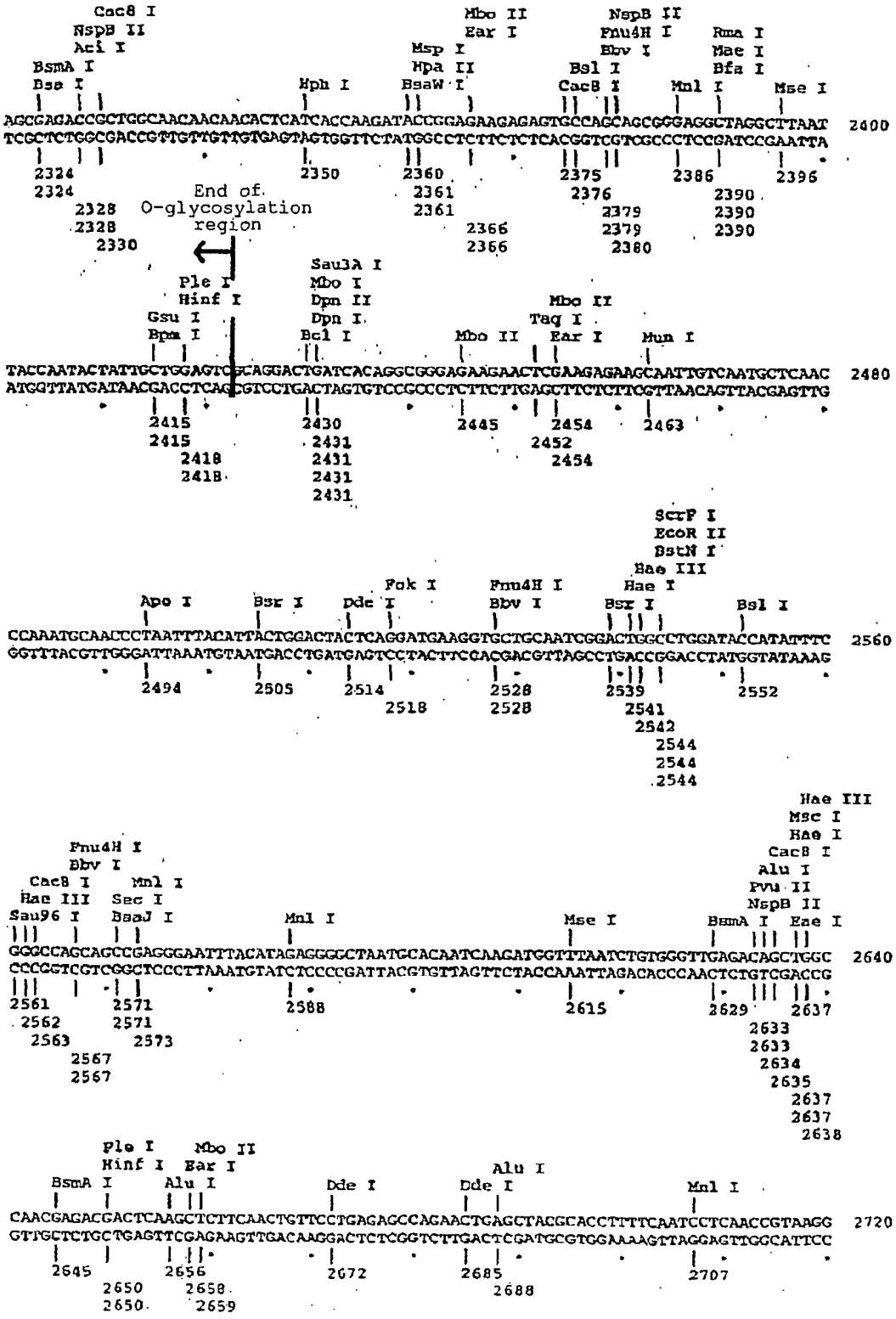
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NspB II
Fnu4H I
Bbv I
Bsl I
CacB I
Rma I
Mae I
Bfa I
Mnl I
Mse I
End of O-glycosylation region
Ple I
Hinf I
Gsu I
Bpm I
Sau3A I
Mbo I
Dpn II
Dpn I
Bcl I
Taq I
Mun I
ScrF I
EcoR II
BstN I
Bae III
Fok I
Apo I
Bsr I
Dde I
Bsr I
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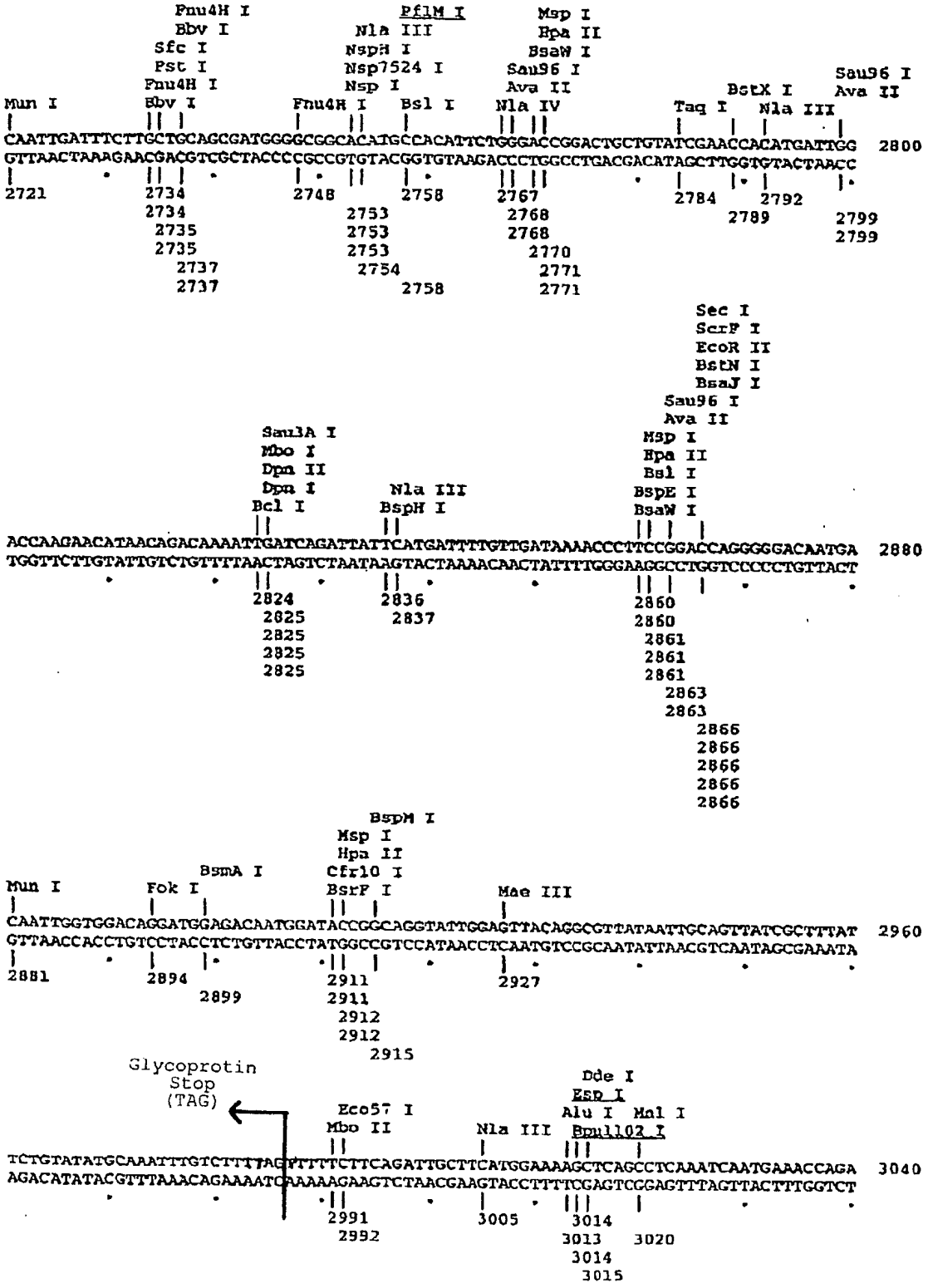
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Bbv I
Sfc I
Pst I
Fnu4H I
Bbv I
Mun I
Nla III
NspH I
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Fnu4H I
Bsl I
PflM I
Msp I
Bpa II
BsaW I
Sau96 I
Ava II
Nla IV
Taq I
BstX I
Nla III
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Ava II
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EcoR II
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BsaJ I
Sau96 I
Ava II
Sau3A I
Mbo I
Dpn II
Dpn I
Bcl I
Nla III
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Msp I
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BspE I
BsaW I
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2825
2825
2825
2836
2837
2860
2860
2861
2861
2861
2863
2863
2866
2866
2866
2866
2866
BspM I
Msp I
Hpa II
BsmA I
Cfr10 I
Mun I
Fok I
BsrF I
Mae III
CAATTGGTGGACAGGATGGAGACAATGGATACCGGCAGGTATTGGAGTTACAGGCGTTATAATTGCAGTTATCGCTTTAT 2960
GTTAACCACCTGTCCTACCTCTGTTACCTATGGCCGTCCATAACCTCAATGTCCGCAATATTAACGTCAATAGCGAAATA
2881
2894
2899
2911
2911
2912
2912
2915
2927
Glycoprotin
Stop
(TAG)
Eco57 I
Mbo II
Nla III
Dde I
ESP I
Alu I
Mnl I
Bpu1102 I
TCTGTATATGCAAATTTGTCTTTTAGTTTTTCTTCAGATTGCTTCATGGAAAAGCTCAGCCTCAAATCAATGAAACCAGA 3040
AGACATATACGTTTAAACAGAAAATCAAAAAGAAGTCTAACGAAGTACCTTTTCGAGTCGGAGTTTAGTTACTTTGGTCT
2991
2992
3005
3013
3014
3014
3015
3020

Fig. 2

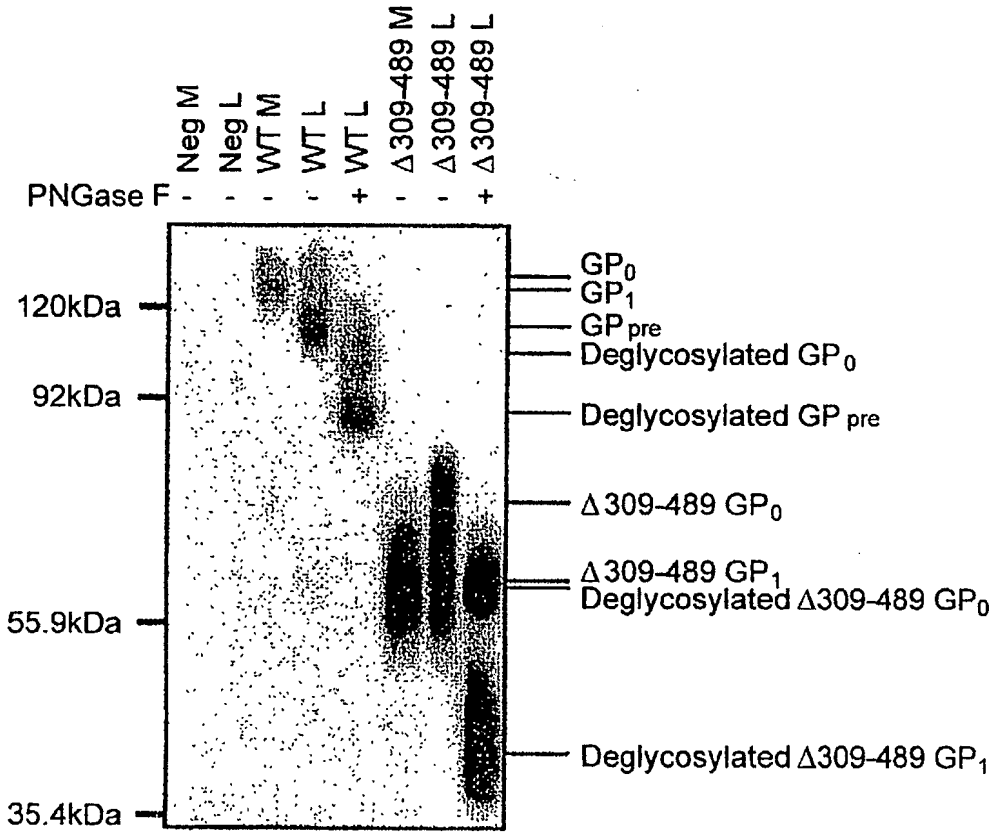
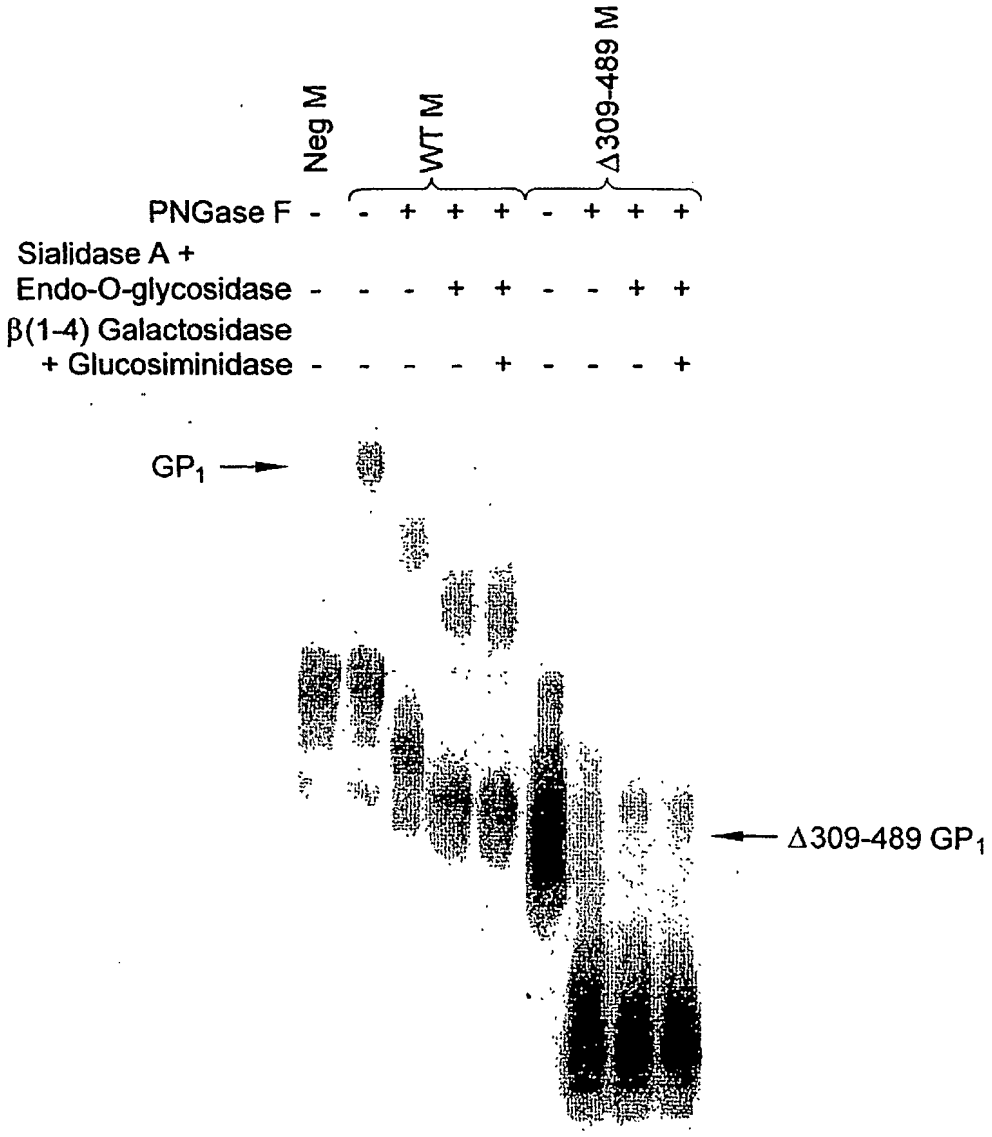

Neg M
Neg L
WT M
WT L
WT L
Δ309-489 M
Δ309-489 L
Δ309-489 L
PNGase F - - - - + - - +
120kDa
92kDa
55.9kDa
35.4kDa
GP0
GP1
GP pre
Deglycosylated GP0
Deglycosylated GP pre
Δ309-489 GP0
Δ309-489 GP1
Deglycosylated Δ309-489 GP0
Deglycosylated Δ309-489 GP1

Fig. 3


Neg M
WT M
Δ309-489 M
PNGase F - - + + + - + + +
Sialidase A +
Endo-O-glycosidase - - - + + - - + +
β(1-4) Galactosidase
+ Glucosiminidase - - - - + - - - +
GP1
Δ309-489 GP1